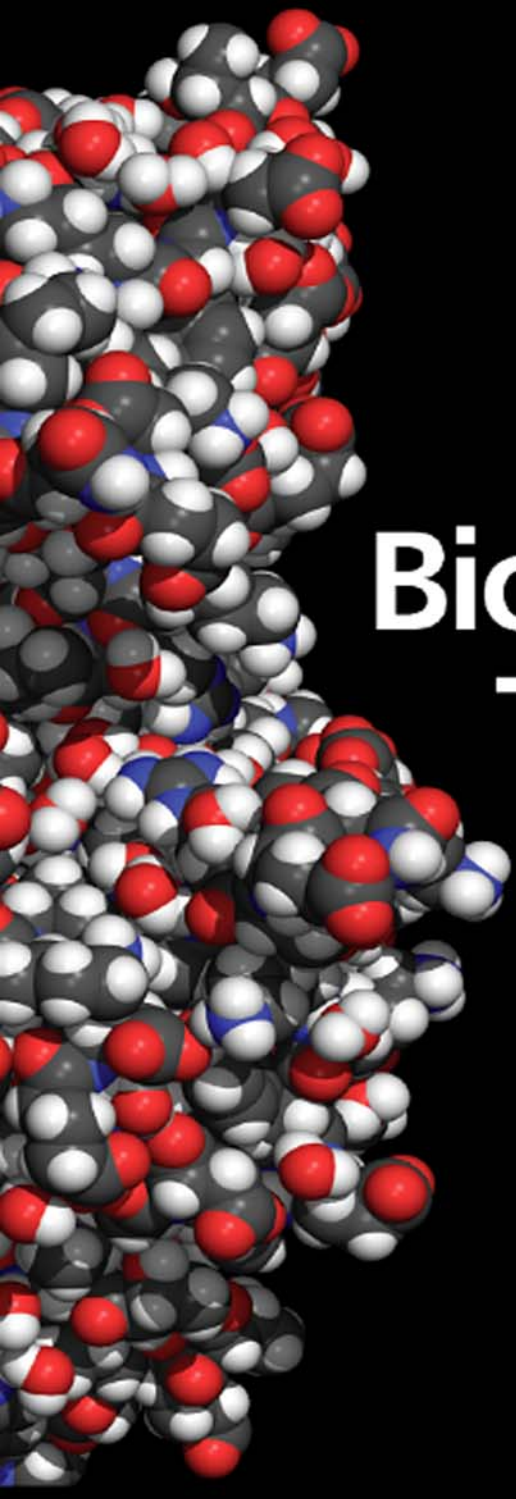
Second Edition



Bioconjugate Techniques

Greg T. Hermanson

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2nd Edition

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2nd Edition

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The molecular model on the cover is a nitrite reductase enzyme obtained from the RCSB Protein Data Bank (2afn), as determined by Murphy, M.E., Turley, S., Kukimoto, M., Nishiyama, M., Horinouchi, S., Sasaki, H., Tanokura, M., and Adman, E.T. (1995) Structure of Alcaligenes faecalis nitrite reductase and a copper site mutant, M150E, that contains zinc. Biochemistry 34, 12107–12117. The space-filling model was created from the coordinate file using PovChem and the final image ray-traced using POV-Ray

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For Amy and Meghan, who, since the first edition was published, have now become interested in pursuing careers in microbiology and medicine.

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Preface to the Second Edition

In the decade since the publication of the first edition, the field of bioconjugation has advanced at an incredible pace. Tens of thousands of additional publications have appeared in the biological, medical, polymer, material science, and chemistry journals describing novel reactions and reagents along with their use in a variety of bioconjugate techniques. In some cases, the innovative application of relatively old organic reactions that now are used to solve new bioconjugation problems has resulted in significant advances in the field. Today, there are more options available than ever before to create nearly any covalent complex imaginable between molecules of virtually any type. In addition, exciting new methods of detecting biomolecules and their interactions have been made possible by recent inventions in bioconjugate chemistry.

Many of the new reactions, reagents, and applications that are featured in this edition didn't even exist at the time that the previous edition was written. For instance, although the preparation of inorganic quantum dots had been described in the physics and material science literature at the time that the first edition was published, their luminescent properties were not applied to biomolecule labeling until only recently. Similarly, the benefits of short hydrophilic polyethylene glycol (PEG)-based spacers in the creation of bioconjugate reagents were mentioned in the first edition, but only within the last few years has a broad range of crosslinkers and modification reagents become available which take advantage of their characteristics.

The recent advances in bioconjugation have resulted in major new sections in this edition, including chapters on Dendrimers and Dendrons; Silane Coupling Agents; Microparticles and Nanoparticles; Buckyballs, Fullerenes, and Carbon Nanotubes; Mass Tags and Isotope Tags; Chemoselective Ligation and Bioorthogonal Reagents; Discrete PEG Compounds; and a chapter on Bioconjugation for the Study of Protein Interactions. In addition, many of the previous chapters now include important additions that include highlights of new reactions and reagents, which reflect the major inventions and innovations made in the field in recent years. For instance, the chapter on Fluorescent Probes now has three new sections: Cyanine Dye Derivatives, Lanthanide Chelates for Time Resolved Fluorescence, and Quantum Dot Nanocrystals. There also are new sections describing protein oxidation reactions, solvent accessibility of functional groups within proteins, and the latest information related to the modification of glycans and other carbohydrates. Many new reagents also are described throughout the updated chapters that were a part of the first edition of the book.

With these new additions comes nearly a doubling of the number of key references cited along with a considerable amount of citation updates throughout the original material. However, the references cited within the book are not designed by any means to be exhaustive for each topic, but rather are intended to provide good starting points for understanding the

concepts and obtaining additional information as needed. For this reason, many review articles are cited along with the first publications describing new reagents or new techniques.

A significant aid in the preparation of the second edition was the tremendous resources now available on the Internet for searching references to virtually any subject or key word within the scientific literature. For this reason, adding endless references to each chapter probably only would increase the size of the book by hundreds of pages, but add very little real value. Far better is for the reader to make use of pertinent Internet databases to search for key words, structure names, or reagent acronyms which can provide lists of hundreds or even thousands of additional references or links regarding any bioconjugation technique of interest.

Some recommended Internet resources for finding bioconjugation-related information include the general Internet search engines like Google or Yahoo in order to obtain a broad spectrum of hits to any bioconjugation topic. This type of search will yield publications, valuable information on web sites dedicated to the desired subject matter, and possible commercial sources for particular reagents. Google Scholar (http://scholar.google.com/) is especially good at finding a broad selection of hits to key words or authors in any field, although its inability to sort the results makes it somewhat limited. In addition, several other dedicated reference databases for science-related topics can be used to complement these general search engines and provide a full spectrum of topical references.

Some Internet search sites that I have found particularly useful include the National Center for Biotechnology information (NCBI) Entrez cross-database search page (http://www.ncbi.nlm.nih.gov/sites/gquery), which includes PubMed Central containing a limited number of free, full text journal articles. In addition, HighWire Press run by Stanford University also contains many free articles from established journals (http://highwire.stanford.edu/) and is able to search the PubMed database simultaneously.

However, some bioconjugation references can't be found in these databases. Some key word searches would yield many additional hits within the chemistry or physical science journals than a search restricted exclusively to the life science journals. For searching within both the life science and physical science journals, perhaps the best option is a multi-database search engine, such as Scirus (http://www.scirus.com). This site is able to search for key words in over 450 million web pages, including all the major science journals. The combined database search can yield many bioconjugation-related references unavailable on the other life science-specific portals. For instance, a search for "dendrimer" on the Entrez cross-database search page returns 1,187 PubMed citations, whereas a Scirus search provides 3,979 hits. The difference relates to the journals that are covered in the database search engine, and the Scirus site accesses the chemistry, polymer, and physics journals inaccessible through PubMed. In addition, Scirus allows searches of any mention of key words on university or institute web pages as well as any other web sites mentioning the specific topic. Including these other sources for a search of "dendrimer" returns a total of 27,708 hits.

Finally, journal web sites and fee-based services can be used with success to find additional references to key topics. Examples of services that are particularly good include the American Chemical Society's Chemical Abstracts Service (CAS; http://www.cas.org/) and their journal search page (http://pubs.acs.org/index.html); the Elsevier Scopus search engine (http://info.scopus.com/) and ScienceDirect database (http://www.sciencedirect.com/); and the ISI Web of Knowledge (http://isiwebofknowledge.com/).

The published procedures that can be found in the journal articles, books, academic web pages, and commercial instruction manuals for particular reagents all formed the basis for

most of the protocols described in this edition. These general methods should be used as starting points for optimizing each conjugation process for a unique application. Often when working with biological molecules like proteins, a method optimized for one protein may need to be adjusted to take into consideration the unique properties of another protein. For instance, it may be simple to conjugate or modify highly soluble proteins that have a high degree of conformational stability. However, similar reactions done on hydrophobic membrane proteins or insoluble peptide sequences often will require changes to the reaction conditions to effect the same conjugation process.

It is my hope that this second edition of Bioconjugate Techniques may stimulate even more ideas, inventions, and innovations and prove useful to scientists in every field who want to take advantage of bioconjugation to create novel tools for research, diagnostics, and therapeutics.

Preface to the First Edition

Bioconjugation involves the linking of two or more molecules to form a novel complex having the combined properties of its individual components. Natural or synthetic compounds with their individual activities can be chemically combined to create unique substances possessing carefully engineered characteristics. Thus, a protein able to bind discretely to a target molecule within a complex mixture may be crosslinked with another molecule capable of being detected to form a traceable conjugate. The detection component provides visibility for the targeting component, producing a complex that can be localized, followed through various processes, or used for measurement.

The technology of bioconjugation has affected nearly every discipline in the life sciences. The application of the available crosslinking reactions and reagent systems for creating novel conjugates with peculiar activities has made possible the assay of minute quantities of substances, the *in vivo* targeting of molecules, and the modulation of specific biological processes. Modified or conjugated molecules have been used for purification, for detection or localization of specific cellular components, and in the treatment of disease.

The ability to chemically attach one molecule to another has caused the birth of billion-dollar industries serving research, diagnostics, and therapeutic markets. A significant portion of all biological assays, including clinical testing, is now done using unique conjugates that have the ability to interact with particular analytes in solutions, cells, or tissues. Crosslinking and modifying agents can be applied to alter the native state and function of peptides and proteins, sugars and polysaccharides, nucleic acids and oligonucleotides, lipids, and almost any other imaginable molecule that can be chemically derivatized. Through careful modification or conjugation strategies, the structure and function of proteins can be investigated, active site conformation discovered, or receptor–ligand interactions revealed. Without the development of bioconjugate chemistry to produce the associated labeled, modified, or conjugated molecules, much of life science research as we know it today would be impossible.

Bioconjugate Techniques attempts to capture the essence of this field through three main sections: its chemistry, reagent systems, and principal applications. Although the scope of bioconjugate technology is enormous, this book provides for the first time a practical overview that condenses this breadth into a single volume. Part I, Bioconjugate Chemistry, begins with a review of the major chemical groups on target molecules that can be used in modification or crosslinking reactions. The chemical reactivities and native properties of proteins, carbohydrates, and nucleic acids are examined in separate chapters, with a view toward designing conjugation strategies that work. Next is a discussion on how to create particular functional groups on these molecules where none exist, or how to transform one chemical group into another. Blocking agents also are examined in this section. The last chapter in Part I summarizes all the major reactions used in bioconjugate chemistry in brief, easy-to-follow descriptions, with liberal references to the literature and to other parts of the book where the reactions are put to use.

Part II, Bioconjugate Reagents, provides a detailed overview organized both by reagent type and by chemical reactivity to present all the major modification and conjugation chemicals commonly used today. The first section in this part examines true crosslinking agents. Zero-length crosslinkers, homobifunctional and heterobi-functional crosslinking agents, and the new trifunctional reagents are discussed with regard to their reactivities, physical properties, and commercial availability. In many cases, conjugation strategies and suggested protocols are presented to illustrate how the reagents may be used in real applications. The next section, Tags and Probes, discusses modification reagents capable of adding fluorescent, radioactive, or biotin labels to molecules. Major fluorophores, including fluorescein, rhodamine, and coumarin derivatives as well as many others, are presented with modification protocols for attaching them to proteins and other molecules. In addition, procedures and compounds for adding radiolabels to molecules, including iodination reagents for ^{125}I-labeling and bifunctional chelating agents to facilitate labeling with other radioisotopes, are discussed. Finally, numerous biotinylation reagents are presented along with protocols for adding a biotin handle to macromolecules for subsequent detection using avidin or streptavidin conjugates.

Part III is by far the largest portion of the book. Bioconjugate Applications discusses how to prepare unique conjugates and labeled molecules for use in particular application areas. This includes: (1) preparing hapten–carrier conjugates for immunization, antibody production, or vaccine research; (2) manufacturing antibody–enzyme conjugates for use in enzyme immunoassay systems; (3) preparing antibody–toxin conjugates for use as targeted therapeutic agents; (4) making lipid and liposome conjugates and derivatives; (5) producing conjugates of avidin or streptavidin for use in avidin–biotin assays; (6) labeling molecules with colloidal gold for sensitive detection purposes; (7) producing polymer conjugates with PEG or dextran to modulate bioactivity or stability of macromolecules; (8) enzyme modification and conjugation strategies; and (9) nucleic acid and oligonucleotide conjugation techniques.

Each of these application areas involves cutting-edge technologies that rely heavily on bioconjugate techniques. In many cases, without the basic ability to attach one molecule to another much of the research progress in these fields would grind to a halt. Bioconjugation thus is not the end but the means to providing the reagent tools necessary to do other research or to produce assays, detection systems, or therapeutic agents.

The purpose of this book is to capture this field in an understandable and practical way, providing the foundation and techniques required to design and synthesize any bioconjugate desired. To aid in this process, over 1,100 pertinent references are cited and over 650 illustrations depicting reactions and chemical compounds are presented. Hundreds of bioconjugate reagents are examined for use in dozens and dozens of potential applications.

The choices available for producing any one conjugate can be overwhelming. I have attempted to identify the best reagents for use in particular application areas, but the presentation is by no means exhaustive. In addition, most of the protocols included in the book are generalized or based on personal experience or literature citations directed at particular applications. Occasionally, applying a bioconjugate protocol that works well in one instance to another application may not work as expected. One or more of the components of the conjugate may lose activity, the conjugate may precipitate, or yields may not be acceptable. In almost every case, some optimization of reaction conditions of reagent choices will have to be done to produce the best possible conjugate or modified molecule for use in a new application. Even protocols as common as antibody–enzyme conjugation techniques may need to be altered somewhat for each new antibody complex produced. The best strategy is to use the suggested protocols, literature citations, and insights gained from this book as starting points to create a bioconjugate that will work well in your own unique application.

Greg T. Hermanson

Acknowledgments

I again acknowledge the large number of scientists who made valuable contributions to the field of bioconjugation in general and to the contents of this book in particular. First, thanks to the thousands of researchers, many of whose names appear in the reference section, which developed and optimized the hundreds of reagents and applications related to the modification and conjugation of biomolecules. I also want to thank Barb Tanaglia, Sally Etheridge, Crystal Gomez, and Heather Flynn for their expert help in obtaining journal references and directing me to the best Internet databases useful for searching the scientific literature. I also thank Craig Smith for reviewing the new material and being so supportive of my writing. Finally, I want to acknowledge David Dellapa, Tom Currier, and Alan Doernberg for giving me corporate approval for this entire endeavor. Although Thermo Fisher Scientific did not sponsor the project, the company provided great motivation for me to undertake the effort and complete the second edition.

Finally, special thanks to the one who made it all possible.

Health and Safety

This book describes hundreds of reagents, reactions, and applications for use in bioconjugation. Most of the compounds are highly specialized and have very little information regarding their toxicological properties. However, the overwhelming majority is known to be reactive and can be corrosive, hazardous, toxic, or dangerous to personal health and safety. At a minimum, bioconjugation reagents should be considered irritants and handled with care. In addition, the disposal of excess reagents or reaction by-products could be harmful to the environment. For this reason, the use of any reagent or protocol described in this book should be done with caution. Refer to the appropriate Material Safety Data Sheet (MSDS) for every compound or component used in a reaction before starting an experiment. The use of personal protective equipment, fume hoods, and proper laboratory techniques can assure safety for both the user and other people in the immediate area.

In addition, the disposal of waste materials should be done according to the appropriate environmental regulations to prevent toxic elements or compounds from entering the water, air, or soil.

It is best to consider every reaction as potentially dangerous and every compound as potentially hazardous (or at least a strong irritant) to avoid injury or damage to health.

Intellectual Property

Throughout this book I have tried to provide the key references to reagents, reactions, and techniques used in bioconjugation. However, such knowledge does not necessarily provide the freedom to legally use them without consideration for existing intellectual property rights. While in some cases, pertinent patent references are provided within the book, this is done only to supply additional technical details about the topic being discussed.

Today, nearly every important reagent or method reported in the literature has a patent or patent application associated with it, especially if it has potential commercial value. A search of the patent databases, such as the United States Patent and Trademark Office (http://www.uspto.gov/) or the European Patent Office (http://ep.espacenet.com/) for key words or the potential names of inventors can provide a list of any existing issued patents or patent applications related to a bioconjugate technique or compound. In addition, a fee-based service such as Delphion is particularly effective at finding patents related to any subject matter (http://www.delphion.com/).

It is the responsibility of the reader to become familiar with patents that may cover particular compounds, compositions, reactions, or their use in bioconjugation applications. If patents or patent applications exist, it is important that permission or a license be obtained to use it before exploiting any intellectual property for commercial use.

PART I

Bioconjugate Chemistry

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1

Functional Targets

Modification and conjugation techniques are dependent on two interrelated chemistries: the reactive functionalities present on the various crosslinking or derivatizing reagents and the functional groups present on the target macromolecules to be modified. Without both types of functional groups being available and chemically compatible, the process of derivatization would be impossible. Reactive functionalities on crosslinking reagents, tags, and probes provide the means to specifically label certain target groups on ligands, peptides, proteins, carbohydrates, lipids, synthetic polymers, nucleic acids, and oligonucleotides. Knowledge of the basic mechanisms by which the reactive groups couple to target functionalities provides the means to intelligently design a modification or conjugation strategy. Choosing the correct reagent systems that can react with the chemical groups available on target molecules forms the basis for successful chemical modification.

The process of designing a derivatization scheme that works well in a given application is not as difficult as it may seem at first glance. A basic understanding of about a dozen reactive functionalities that are commonly present on modification and crosslinking reagents combined with knowledge of about half that many functional target groups can provide the minimum skills necessary to plan a successful experiment.

Fortunately, the principal reactive functionalities commonly encountered on bioconjugate reagents are now present on scores of commercially obtainable compounds. The resource that this arsenal of reagents provides can assist in solving almost any conceivable modification or conjugation problem. The following sections describe the predominant targets for these reagent systems. The functionalities discussed are found on virtually every conceivable biological molecule, including amino acids, peptides, proteins, sugars, carbohydrates, polysaccharides, nucleic acids, oligonucleotides, lipids, and complex organic compounds. A careful understanding of target molecule structure and reactivity provides the foundation for the successful use of all of the modification and conjugation techniques discussed in this book.

1. Modification of Amino acids, Peptides, and Proteins

Protein molecules are perhaps the most common targets for modification or conjugation techniques. As the mediators of specific activities and functions within living organisms, proteins can be used *in vitro* and *in vivo* to effect certain tasks. Having enough of a protein that can bind a

particular target molecule can result in a way to detect or assay the target providing the protein can be followed or measured. If such a protein doesn't possess an easily detectable component, it often can be modified to contain a chemical or biological tracer to allow detectability. This type of protein complex can be designed to retain its ability to bind its natural target, while the tracer portion can provide the means to find and measure the location and amount of target molecules.

Detection, assay, tracking, or targeting of biological molecules by using the appropriately modified proteins are the main areas of application for modification and conjugation systems. The ability to produce a labeled protein having specificity for another molecule provides the key component for much of biological research, clinical diagnostics, and human therapeutics.

In this section, the structure, function, and reactivity of amino acids, peptides, and proteins will be discussed with the goal of providing a foundation for successful derivatization. The interplay of amino acid functionality and the three-dimensional folding of polypeptide chains will be seen as forming the basis for protein activity. Understanding how the attachment of foreign molecules can affect this tenuous relationship, and thus alter protein function, ultimately will create a rational approach to protein chemistry and modification.

1.1. Protein Structure and Reactivity

Amino Acids

Peptides and proteins are composed of amino acids polymerized together through the formation of peptide (amide) bonds. The peptide bonded polymer that forms the backbone of polypeptide structure is called the α-chain. The peptide bonds of the α-chain are rigid planar units formed by the reaction of the α-amino group of one amino acid with the α-carboxyl group of another (Figure 1.1). The peptide bond possesses no rotational freedom due to the partial double bond character of the carbonyl-amino amide bond. The bonds around the α-carbon atom, however, are true single bonds with considerable freedom of movement.

The sequence and properties of the amino acid constituents determine protein structure, reactivity, and function. Each amino acid is composed of an amino group and a carboxyl group bound to a central carbon, termed the α-carbon. Also bound to the α-carbon are a hydrogen atom and a side chain unique to each amino acid (Figure 1.2). There are 20 common amino acids found throughout nature, each containing an identifying side chain of particular chemical structure, charge, hydrogen bonding capability, hydrophilicity (or hydrophobicity), and reactivity.

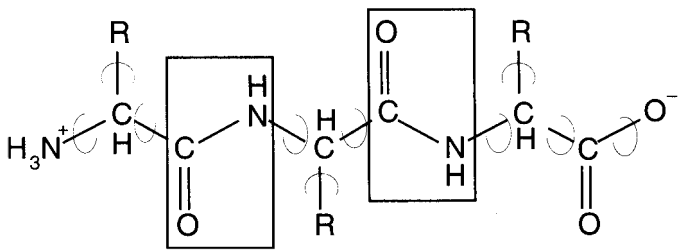


Figure 1.1 Rigid peptide bonds link amino acid residues together to form proteins. Other bonds within the polypeptide structure may exhibit considerable freedom of rotation.

The side chains do not participate in polypeptide formation and are thus free to interact and react with their environment.

Amino acids may be grouped by type depending on the characteristics of their side chains. There are seven amino acids that contain aliphatic side chains, which are relatively non-polar and hydrophobic: glycine, alanine, valine, leucine, isoleucine, methionine, and proline (Figure 1.3). Glycine is the simplest amino acid—its side chain consisting of only a hydrogen atom. Alanine is next in line, possessing just a single methyl group for its side chain. Valine, leucine, and isoleucine are slightly more complex with three or four carbon branched-chain constituents. Methionine is unique in that it is the only reactive aliphatic amino acid, containing a thioether group at the terminus of its hydrocarbon chain. Proline is actually the only *imino* acid. Its side chain forms a pyrrolidine ring structure with its α-amino group. Thus, it is the only amino acid containing a secondary α-amine. Due to its unique structure, proline often causes severe turns in a polypeptide chain. Proteins rich in proline, such as collagen, have tightly formed structures of high density. Collagen also contains a rare derivative of proline, 4-hydroxyproline, found in only a few other proteins. Proline, however, cannot be accommodated in normal α-helical structures, except at the ends where it may create the turning point for the chain. Poly-proline α-helical structures have

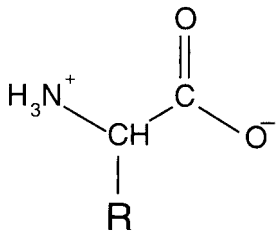


Figure 1.2 Individual amino acids consist of a primary (α) amine, a carboxylic acid group, and a unique side-chain structure (R). At physiological pH, the amine is protonated and bears a positive charge, while the carboxylate is ionized and possesses a negative charge.

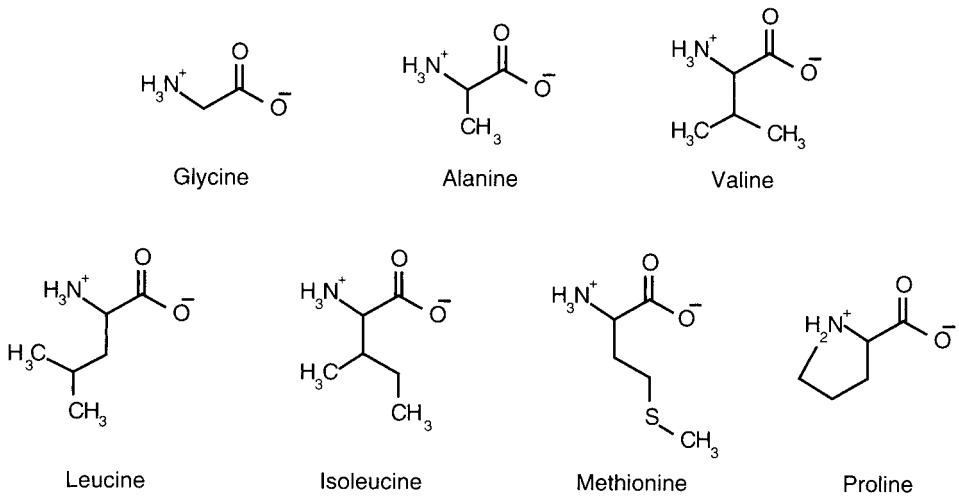


Figure 1.3 Common aliphatic amino acids.

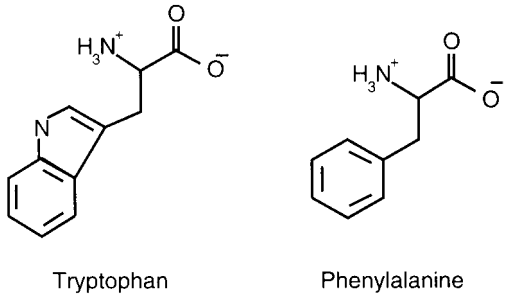


Figure 1.4 The two non-polar aromatic amino acids.

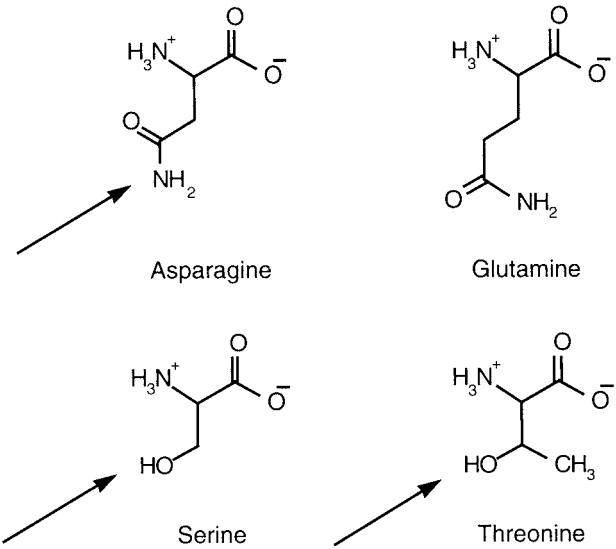


Figure 1.5 The four polar amino acids. The arrows show the attachment points for carbohydrate residues on glycoproteins.

been formed, but the structural characteristics of these artificial polypeptides are quite different from native protein helices.

Phenylalanine and tryptophan contain aromatic side chains that, like the aliphatic amino acids, are also relatively non-polar and hydrophobic (Figure 1.4). Phenylalanine is unreactive toward common derivatizing reagents, whereas the indolyl ring of tryptophan is quite reactive, if accessible. The presence of tryptophan in a protein contributes more to its total absorption at 275–280 nm on a mole-per-mole basis than any other amino acid. The phenylalanine content, however, adds very little to the overall absorbance in this range.

All of the aliphatic and aromatic hydrophobic residues often are located at the interior of protein molecules or in areas that interact with other non-polar structures such as lipids. They usually form the hydrophobic core of proteins and are not readily accessible to water or other hydrophilic molecules.

There is another group of amino acids that contains relatively polar constituents and are thus hydrophilic in character. Asparagine, glutamine, threonine, and serine (Figure 1.5) are

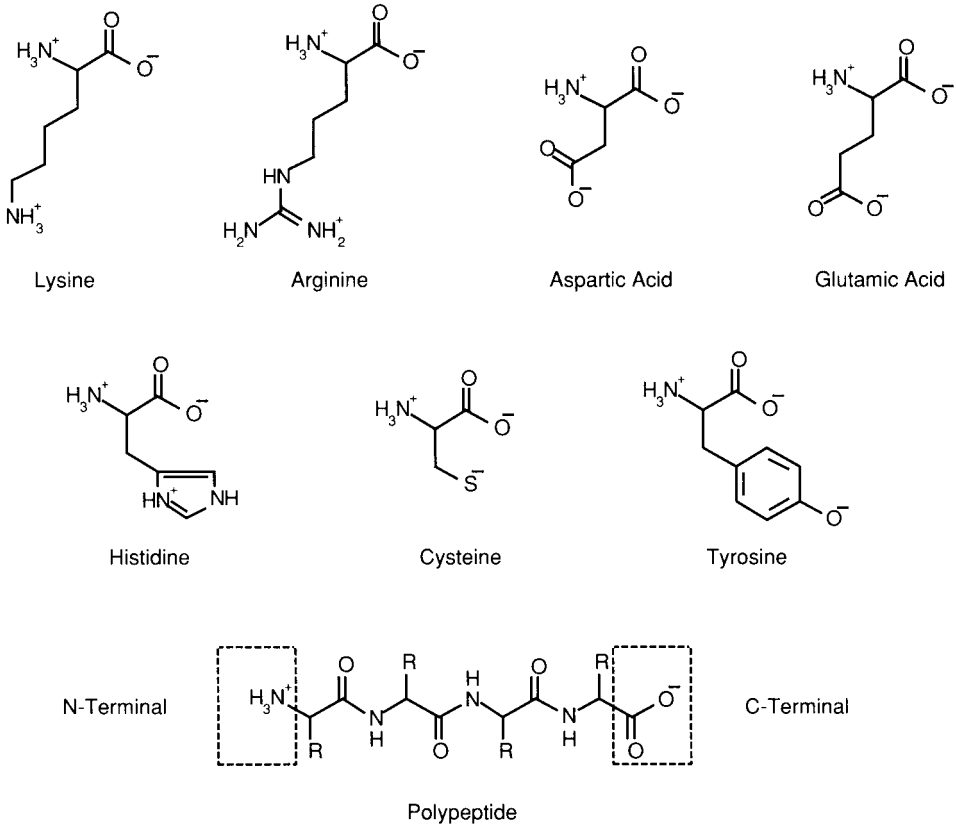


Figure 1.6 The ionizable amino acids possess some of the most important side-chain functional groups for bioconjugate applications. The C- and N-terminal of each polypeptide chain also is included in this group.

usually found in hydrophilic regions of a protein molecule, especially at or near the surface where they can be hydrated with the surrounding aqueous environment. Asparagine, threonine, and serine often are found post-translationally modified with carbohydrate in *N*-glycosidic (asp) and *o*-glycosidic linkages (threonine and serine). Though these side chains are enzymatically derivatized in nature, the hydroxyl and amide portions have relatively the same nucleophilicity as that of water and are therefore difficult to modify with common reagent systems under aqueous conditions.

The most significant amino acids for modification and conjugation purposes are the ones containing ionizable side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, and tyrosine (Figure 1.6). In their unprotonated state, each of these side chains can be potent nucleophiles to engage in addition reactions (see the discussion on nucleophilicity below).

Both aspartic and glutamic acids contain carboxylate groups that have similar ionization properties to the C-terminal α-carboxylate. The theoretical pK_a of the β-carboxyl of aspartic acid (3.7–4.0) and the γ-carboxyl of glutamic acid (4.2–4.5) are somewhat higher than the α-carboxyl groups at the C-terminal of a polypeptide chain (2.1–2.4). At pH values above their pK_a, these groups are generally ionized to negatively charged carboxylates. Thus at physiological

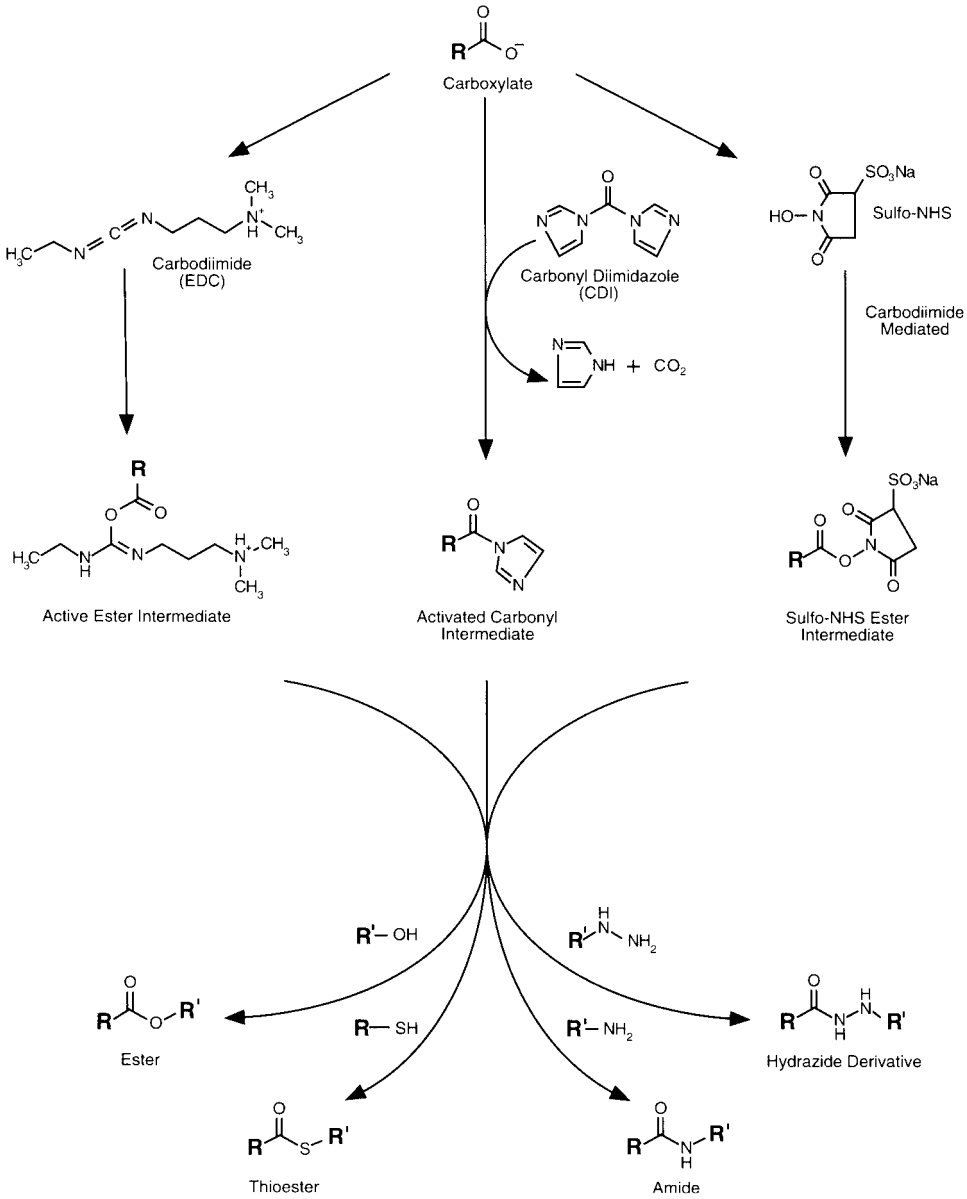


Figure 1.7 Derivatives of carboxylic acids can be prepared through the use of active intermediates that react with target functional groups to give acylated products.

pH, they contribute to the overall negative charge contribution of an intact protein (see following section).

Carboxylate groups in proteins may be derivatized through the use of amide bond forming agents or through active ester or reactive carbonyl intermediates (Figure 1.7). The carboxylate actually becomes the acylating agent to the modifying group. Amine containing nucleophiles

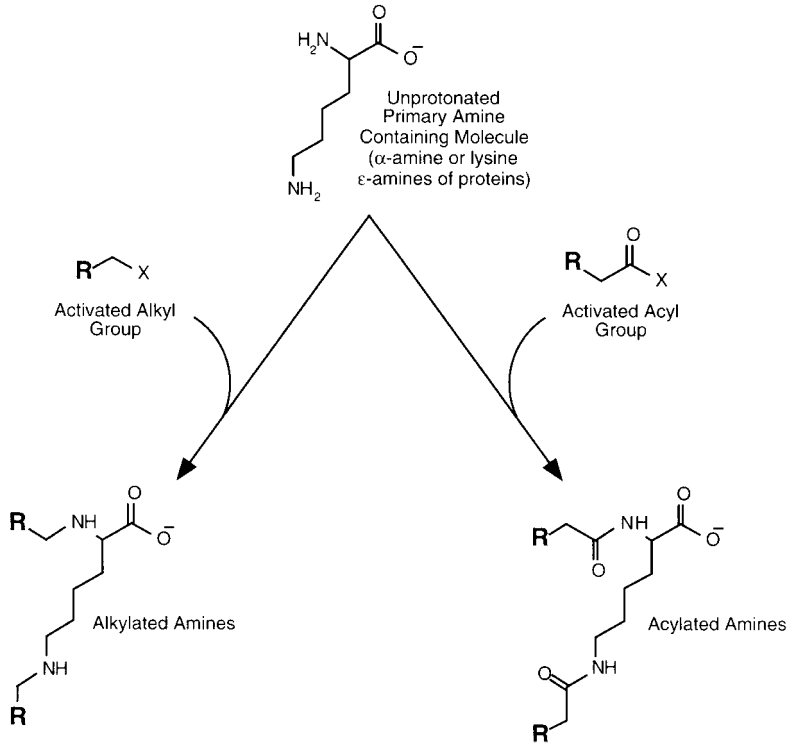


Figure 1.8 Derivatives of amines can be prepared from acylating or alkylating agents to give amide, secondary amine, or tertiary amine bonds.

can couple to an activated carboxylate to give amide derivatives. Hydrazide compounds react similarly to amines. Sulfhydryls, while reactive and resulting in a thioester linkage, form relatively unstable derivatives, which can exchange with other nucleophiles such as amines or hydrolyze in aqueous solutions.

Lysine, arginine, and histidine have ionizable amine containing side chains that, along with the N-terminal α-amine, contribute to a protein's overall net positive charge. Lysine contains a straight four-carbon chain terminating in a primary amine group. The ε-amine of lysine differs in pK_a from the primary α-amines pK_a by having a slightly higher ionization point (pK_a of 9.3–9.5 for lysine versus pK_a of 7.6–8.0 for α-amines). At pH values lower than the pK_a of these groups, the amines are generally protonated and possess a positive charge. At pH values greater than the pK_a, the amines are unprotonated and contribute no net charge. Arginine contains a strongly basic chemical constituent on its side chain called a guanidino group. The ionization point of this residue is so high ($pK_a > 12.0$) that it is virtually always protonated and carries a positive charge. Histidine's side chain is an imidazole ring that is potentially protonated at slightly acidic pH values ($pK_a = 6.7–7.1$). Thus, at physiological pH, these residues contribute to the overall net positive charge of an intact protein molecule.

The amine containing side chains in lysine, arginine, and histidine typically are exposed on the surface of proteins and can be derivatized with ease. The most important reactions that can occur with these residues are alkylation and acylation (Figure 1.8). In alkylation, an active

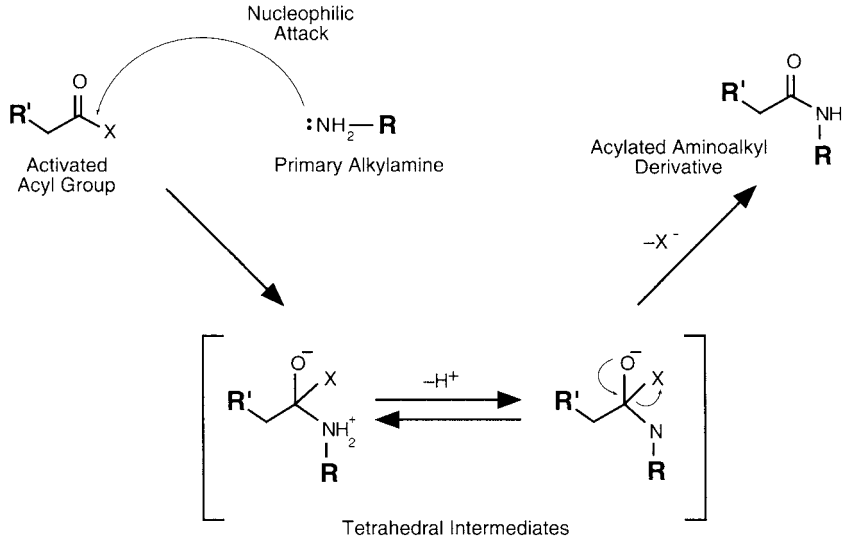


Figure 1.9 The mechanism of acylation proceeds through the attack of a nucleophile, generating a tetrahedral intermediate, which then goes on to form the product.

alkyl group is transferred to the amine nucleophile with loss of one hydrogen. In acylation, an active carbonyl group undergoes addition to the amine. Alkylating reagents are highly varied and the reaction with an amine nucleophile is difficult to generalize. Acylating reagents, however, usually proceed through a carbonyl addition mechanism as shown in Figure 1.9. The imidazole ring of histidine also is an important reactive species in electrophilic reactions, such as in iodination using radioactive ^{125}I or ^{131}I (Chapter 12).

Cysteine is the only amino acid containing a sulfhydryl group. At physiological pH, this residue is normally protonated and possesses no charge. Ionization only occurs at high pH (pK_a = 8.8–9.1) and results in a negatively charged thiolate residue. The most important reaction of cysteine groups in proteins is the formation of disulfide crosslinks with another cysteine molecule. Cysteine disulfides (called cystine residues) often are key points in stabilizing protein structure and conformation. They frequently occur between polypeptide subunits, creating a covalent linkage to hold two chains together. Cysteine and cystine groups are relatively hydrophobic and usually can be found within the core of a protein. For this reason, it is often difficult to fully reduce the disulfides of large proteins without a deforming agent present to open up the inner structure and make them accessible (see Chapter 1, Section 4.1).

Cysteine sulfhydryls and cystine disulfides may undergo a variety of reactions, including alkylation to form stable thioether derivatives, acylation to form relatively unstable thioesters, and a number of oxidation and reduction processes (Figure 1.10). Derivatization of the side chain sulfhydryl of cysteine is one of the most important reactions of modification and conjugation techniques for proteins.

Tyrosine contains a phenolic side chain with a pK_a of about 9.7–10.1. Due to its aromatic character, tyrosine is second only to tryptophan in contributing to a protein's overall absorptivity at 275–280 nm. Although the amino acid is only sparingly soluble in water, the ionizable nature of the phenolic group makes it often appear in hydrophilic regions of a protein—usually

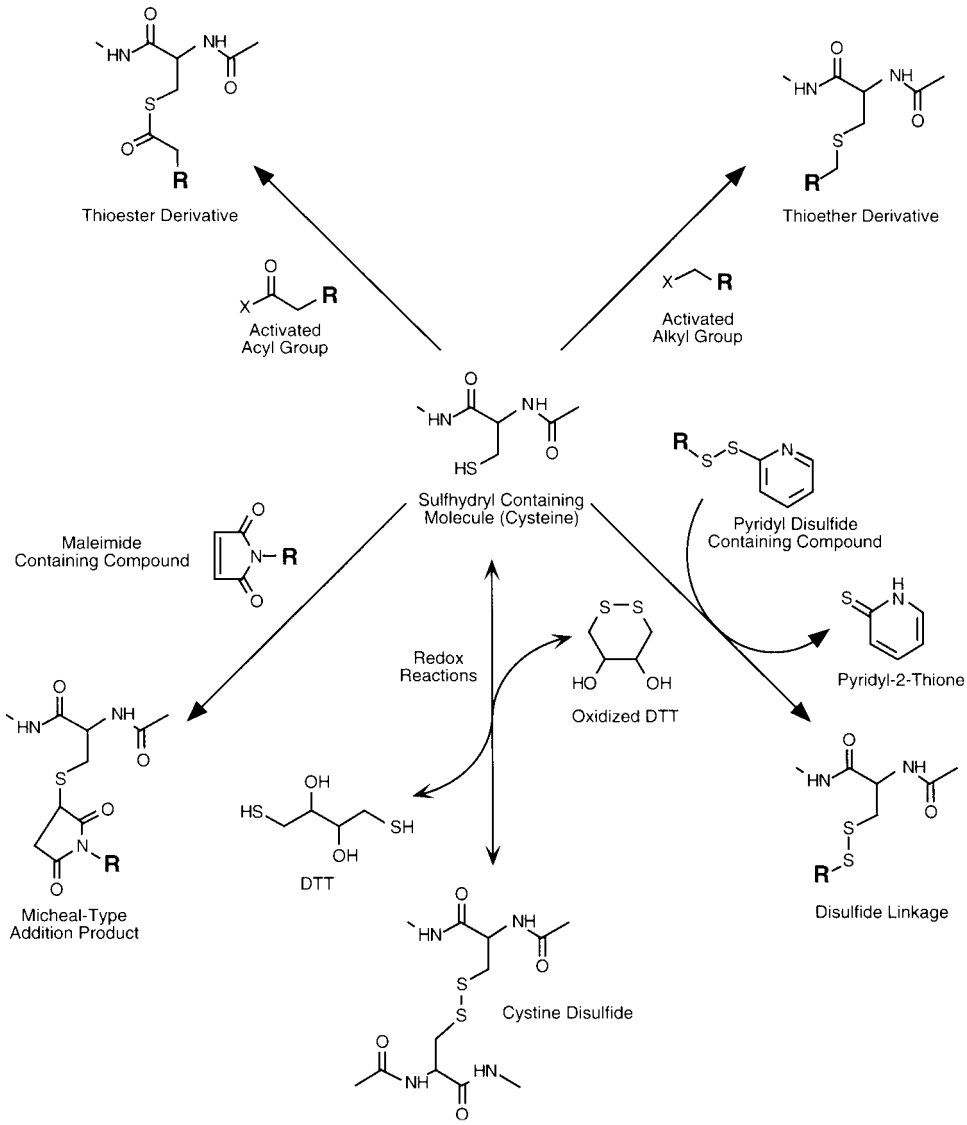


Figure 1.10 Sulfhydryl groups may undergo a number of additional reactions, including acylation and alkylation. Thiols also may participate in redox reactions, which generate reversible disulfide linkages.

at or near the surface. Thus tyrosine derivatization proceeds without much need for deforming agents to further open protein structure.

Tyrosine may be targeted specifically for modification through its phenolate anion by acylation, through electrophilic reactions such as the addition of iodine or diazonium ions, and by Mannich condensation reactions. The electrophilic substitution reactions on tyrosine's ring all occur at the *ortho* position to the —OH group (Figure 1.11). Most of these reactions proceed effectively only when tyrosine's ring is ionized to the phenolate anion form.

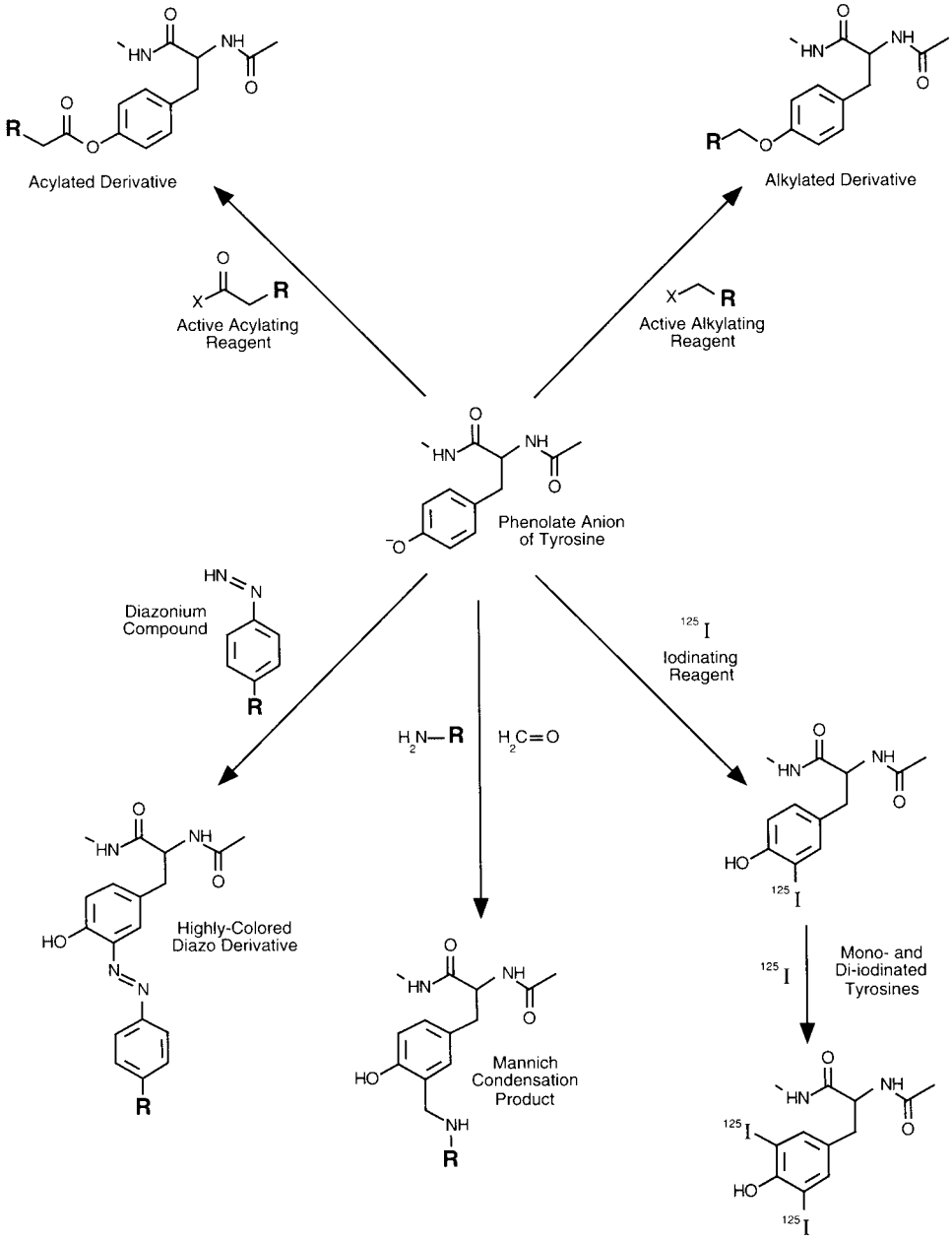


Figure 1.11 Tyrosine residues are subject to nucleophilic and electrophilic reactions. The unprotonated phenolate ion may be alkylated or acylated using a variety of bioconjugate reagents. Its aromatic ring also may undergo electrophilic addition using diazonium chemistry or Mannich condensation, or be halogenated with radioactive isotopes such as ^{125}I.

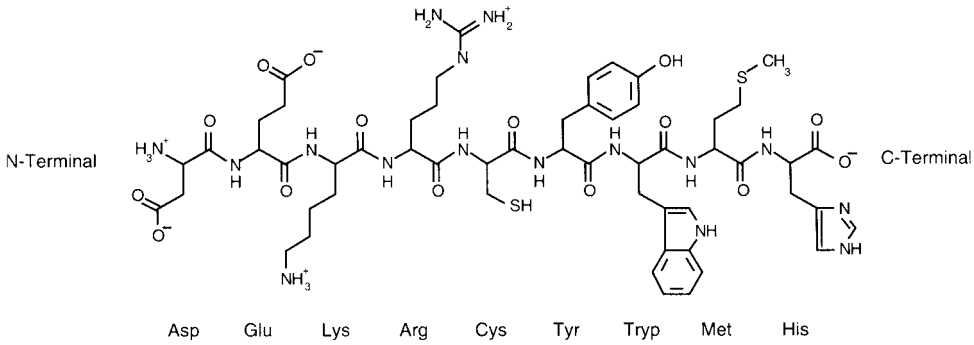


Figure 1.12 The more important polypeptide functional groups are represented by these nine amino acids. Bioconjugate chemistry may occur through the C- and N-terminals of each polypeptide chain, the carboxylate groups of aspartic and glutamic acids, the ε-amine of lysine, the guanidino group of arginine, the sulfhydryl group of cysteine, the phenolate ring of tyrosine, the indol ring of tryptophan, the thioether of methionine, and the imidazole ring of histidine.

In summary, protein molecules may contain up to nine amino acids that are readily derivatizable at their side chains: aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, tyrosine, methionine, and tryptophan. These nine residues contain eight principal functionalities with sufficient reactivity for modification reactions: primary amines, carboxylates, sulfhydryls (or disulfides), thioethers, imidazolyls, guanidinyl groups, and phenolic and indolyl rings. All of these side chain functionalities in addition to the N-terminal α-amino and the C-terminal α-carboxylate form the full complement of polypeptide reactivity within proteins (Figure 1.12).

Nucleophilic Reactions and the pI of Amino Acid Side Chains

Ionizable groups within proteins can exist in one of two forms: protonated or unprotonated. Carboxylate groups below their pK_a values exist in the protonated state and are therefore in the conjugate acid form and carry no charge. However, at pH values above the pK_a of the carboxylic group, the acid is ionized and therefore unprotonated to a negative charge. This same relationship is true of the —OH group on the phenol ring of tyrosine. At pH values below its pK_a, tyrosine's side chain is uncharged. Above the pK_a, however, the hydrogen ionizes off leaving a negatively charged phenolate. Conversely, amine nucleophiles below their pK_a values are in a protonated state and possess a positive charge. At pH values above the pK_a of the amino group, it is then ionized and unprotonated to neutrality.

Each type of ionizable group in proteins will have a unique pK_a based upon the theoretical value for the amino acid and modulated from that value by its own surrounding microenvironment. Minute environmental changes will cause amine containing residues at different structural locations to have different ionization potentials, even if the groups are otherwise chemically identical.

Thus, the actual pK_a of each ionizable group within protein molecules may range considerably lower or higher than the theoretical values as the microenvironment of individual groups changes. Identical side chains in different parts of a protein molecule may have widely varying pK_a values depending on the immediate chemical milieu. Such factors as the presence of other amino acid side chains in the vicinity, salts, buffers, temperature, ionic strength, and other effects of the

solvent medium all play crucial roles in creating microenvironmental changes that affect the ionization potential of these groups (Tanford and Hauenstein, 1956; Schewale and Brew, 1982).

The Henderson–Hasselbalch equation (1.1) explains the relationship of pH and pK_a to the relative ratios of protonated (acid) and unprotonated (base) forms of an ionizable group. Note that the ionized form of such a group does not have to possess a negative charge, as in the case of unprotonated primary amines. Indeed, in that instance it is the protonated amine that bears a charge of positive one. According to the mathematical implications of this equation, an ionizable group at its pK_a value is exactly 50 percent ionized. This means that aspartic acid side chains placed in a medium with a pH equal to its pK_a should have half of its carboxylates ionized to a negative charge and half of them unionized with no charge.

$$pH = pK_a + \log\{[\text{base}]/[\text{acid}]\} \tag{1.1}$$

Further implications of this equation are that at one pH unit below or above the pK_a, an ionizable group will be 91 percent unionized (protonated) or 91 percent ionized (unprotonated), respectively. Two pH units below or above translate to a 99 percent unionized or 99 percent ionized state.

The absolute ratio of protonated-to-unprotonated forms will change from this theoretical approach based upon the microenvironment each group experiences. The reactivity of amino acid side chains is directly related to them being in an unprotonated or ionized state. Many reactions of modification and conjugation occur efficiently only when the nucleophilic species is in an ionized form. As the unprotonated form increases in concentration, the relative nucleophilicity of the ionizable group increases. Many of the reactive groups commonly used for protein modification will couple in greater yield as the pH of the reaction is raised closer to the pK_a of the ionizable target. However, continuing to increase the pH beyond the pK_a may not be necessary for increased yield, and may even be detrimental, because many reactive groups will begin to loose activity through hydrolysis at high pHs.

A nucleophile is any atom containing an unshared pair of electrons or an excess of electrons able to participate in covalent bond formation. Nucleophilic attack at an atomic center of electron deficiency or positive charge is the basis for many of the coupling reactions that occur in chemical modification. Thus, an uncharged amine group is a more powerful nucleophile than the protonated form bearing a positive charge. Likewise, a negatively charged carboxylate has greater nucleophilicity than its uncharged, protonated conjugate acid form. In addition, an unprotonated thiolate, bearing a negative charge (RS^-), is a much more powerful nucleophile than its protonated, uncharged sulfhydryl form.

According to the theory of nucleophilicity (Edwards and Pearson, 1962; Bunnett, 1963; Pearson *et al.*, 1968), the relative order of nucleophilicity relative to the major groups in biological molecules can be summarized as follows:

$$R{-}S^- > R{-}SH$$

$$R{-}NH_2 > R{-}NH_3^+$$

$$R{-}COO^- > R{-}COOH$$

$$R{-}O^- > R{-}OH$$

$$R{-}OH = H{-}OH$$

and finally,

$$R—S^- > R—NH_2 > R—COO^- = R—O^-$$

Using these relationships, it is obvious that the strongest nucleophile in protein molecules is the sulfhydryl group of cysteine, particularly in the ionized, thiolate form. Next in line are the amine groups in their uncharged, unprotonated forms, including the α-amines at the N-terminals, the ε-amines of lysine side chains, the secondary amines of histidine imidazolyl groups and tryptophan indol rings, and the guanidino amines of arginine residues. Finally, the least potent nucleophiles are the oxygen containing ionizable groups including the α-carboxylate at the C-terminal, the β-carboxyl of aspartic acid, the γ-carboxyl of glutamic acid, and the phenolate of tyrosine residues.

According to the theoretical pK_a values for the ionizable side chains of amino acids, nucleophilic substitution reactions involving primary amines or sulfhydryl groups on proteins should not be efficient below a pH of about 8.5 (Table 1.1). In practice, however, reactions can be done with these groups in high yield at pH values not much higher than neutrality. This discrepancy relates to the changes in pK_a due to microenvironmental effects experienced by the residues within the three-dimensional structure of the protein molecule. In reality, the ε-amine groups on lysine side chains within proteins, having theoretical pK_as of over 10, nonetheless exist in sufficient quantity in an unprotonated form even at a pH of 7.2 that modification easily occurs.

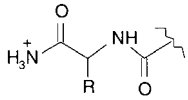
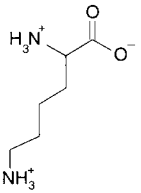
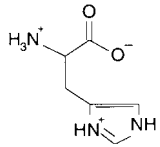
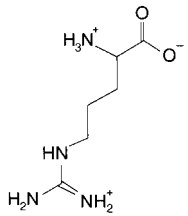
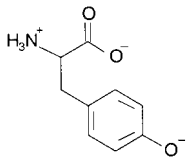
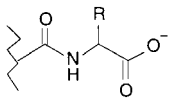
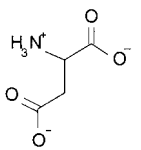
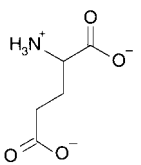
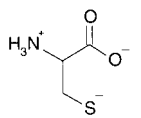
One important point should be noted, however. The changes that occur in the pK_a of ionizable groups in protein molecules due to microenvironmental effects sometimes make it difficult to select certain residues for modification simply by careful modulation of reaction pH. For instance, at least in theory, overlap of the pK_a range for sulfhydryls and amine-containing residues would eliminate any chance of directing a reaction toward —SH groups solely by adjusting the pH of the reaction medium. However, because of the microenvironmental changes that occur in complex biomolecules, pH sometimes can be used along with the right reactive group to target thiols without amine modification. Thus, in practice, to effectively site-direct a modification reaction, the proper choice of reactive group and reaction conditions can result in highly discrete conjugation to certain sites within proteins.

Secondary, Tertiary, and Quaternary Structure

Amino acids are linked through peptide bonds to form long polypeptide chains. The *primary* structure of protein molecules is simply the linear sequence of each residue along the α-chain. Each amino acid in the chain interacts with surrounding groups through various weak, noncovalent interactions and through its unique side chain functionalities. Noncovalent forces such as hydrogen bonding and ionic and hydrophobic interactions combine to create each protein's unique organization.

It is the sequence and types of amino acids and the way that they are folded that provides protein molecules with specific structure, activity, and function. Ionic charge, hydrogen bonding capability, and hydrophobicity are the major determinants for the resultant three-dimensional structure of protein molecules. The α-chain is twisted, folded, and formed into globular structures, α-helicies, and β-sheets based upon the side-chain amino acid sequence and weak intramolecular interactions such as hydrogen bonding between different parts of the peptide

Table 1.1 pK_a of Ionizable Amino Acids

Group location	Functionality	pK_a range
α-Amine; N-Terminus		7.6–8
Lysine's ε-amine		9.3–9.5
Histidine's imidazolyl nitrogen		6.7–7.1
Arginine's guanidinyl group		>12
Tyrosine's phenolic hydroxyl		9.7–10.1
α-Carboxyl; C-terminus		2.1–2.4
Aspartic acid's γ-carboxyl		3.7–4
Glutamic acid's γ-carboxyl		4.2–4.5
Cysteine's sulfhydryl		8.8–9.1

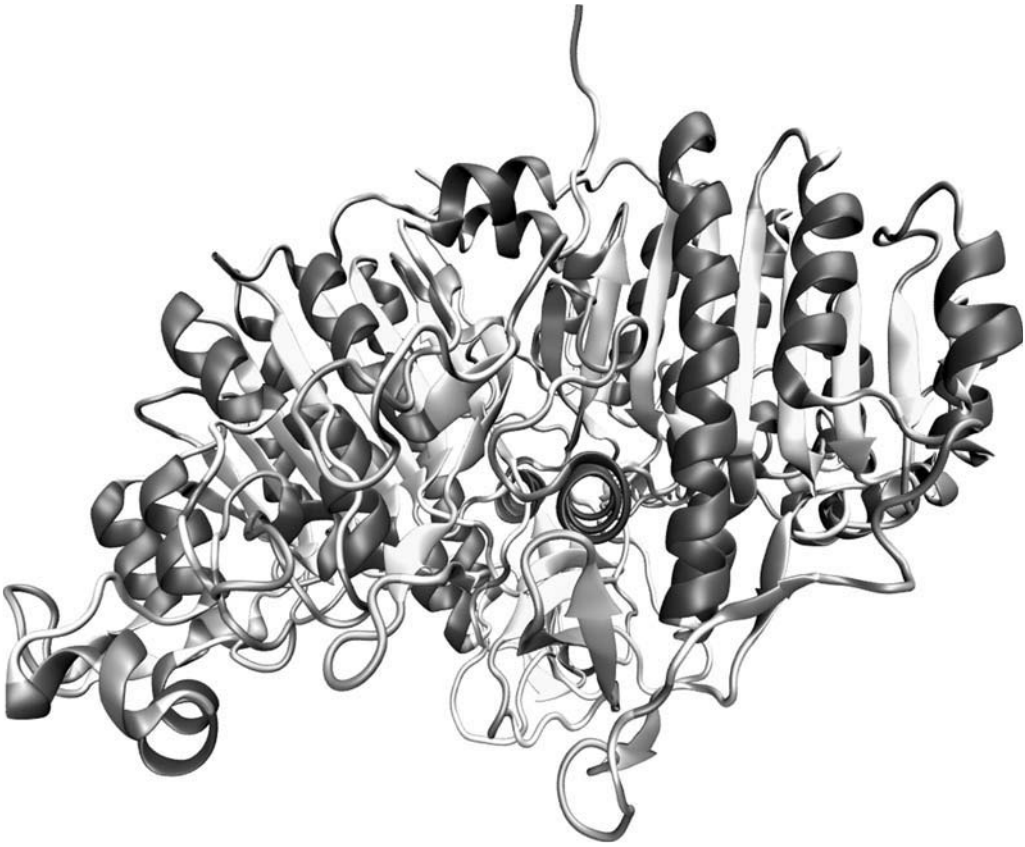


Figure 1.13 The α-chain structure of alkaline phosphatase illustrates the complex nature of polypeptide structure within proteins (Kim and Wyckoff, 1991).

backbone (Figure 1.13). Major secondary structures of proteins such as α-helicies and β-sheets are held together solely by massive hydrogen bonding created through the carbonyl oxygens of peptide bonds interacting with the hydrogen atoms of other peptide bonds (Figure 1.14).

In addition, negatively charged residues may become bonded to positively charged groups through ionic interactions. Non-polar side chains may attract other non-polar residues and form regions of hydrophobicity to the exclusion of water and other ionic groups. Occasionally, disulfide bonds also are found holding different regions of the polypeptide chain together. All of these forces combine to create the *secondary* structure of proteins, which is the way the polypeptide chain folds in local areas to form larger, sometimes periodic structures.

On a larger scale, the unique folding and structure of one complete polypeptide chain is termed the *tertiary* structure of protein molecules. The difference between local secondary structure and complete polypeptide tertiary structure is arbitrary and sometimes of little practical difference.

Larger proteins often contain more than one polypeptide chain. These multi-subunit proteins have a more complex shape, but are still formed from the same forces that twist and fold

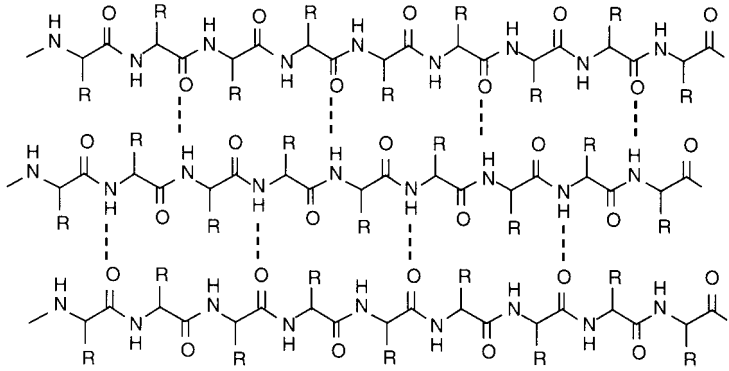


Figure 1.14 Secondary structure within proteins may be stabilized through hydrogen bonding between adjacent α-chains, forming β-sheet conformations.

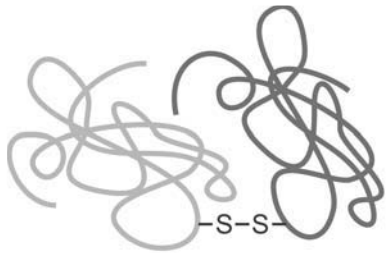


Figure 1.15 Polypeptide chains may be bound together through disulfide linkages occurring between cysteine residues within each subunit.

the local polypeptide. The unique three-dimensional interaction between different polypeptides in multi-subunit proteins is called the *quaternary* structure. Subunits may be held together by noncovalent contacts, such as hydrophobic or ionic interactions, or by covalent disulfide bonds formed from the cysteine residue of one polypeptide chain being crosslinked to a cysteine sulfhydryl of another chain (Figure 1.15).

Thus, aside from the covalently polymerized α-chain itself, the majority of protein structure is determined by weaker, noncovalent interactions that potentially can be disturbed by environmental changes. It is for this reason that protein structure can be easily disrupted or denatured by fluctuations in pH, temperature, or by substances that can alter the structure of water, such as detergents or chaotropes.

Not surprisingly, chemical modification to the amino acid constituents of a polypeptide chain also may cause significant disruption in the overall three-dimensional structure of a protein. If amino acid residues critical to folding near functionally important regions are modified with chemical groups that change the charge, hydrophilicity, or hydrogen bonding character of the polypeptide chain, protein structure may be altered and activity may be compromised. This concept will be discussed further in subsequent sections.

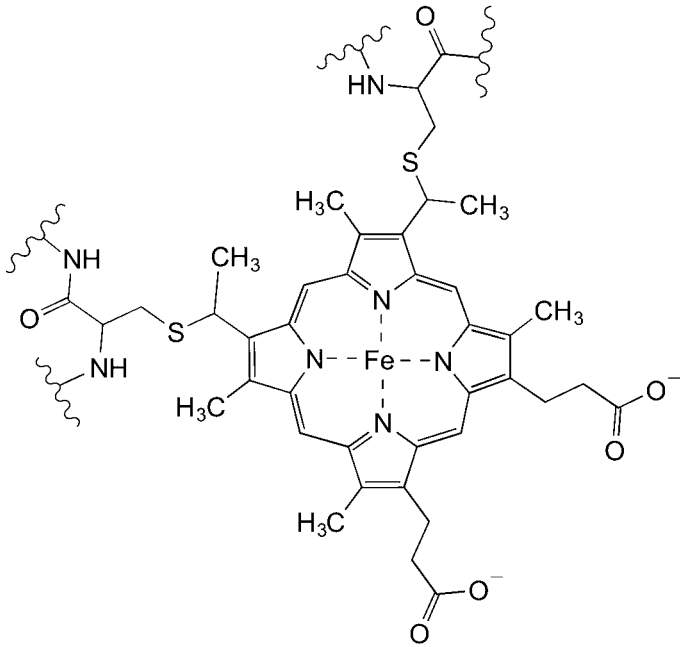


Figure 1.16 The heme ring of cytochrome C is a non-amino acid, prosthetic group bound to the protein through two cysteine residues.

Prosthetic Groups, Cofactors, and Post-Translational Modifications

Proteins may contain structures other than polypeptide chains that are important for biological function. Prosthetic groups and cofactors are small organic compounds that are sometimes tightly bound to a protein and aid in forming the active center. A prosthetic group is usually carried within the three-dimensional protein structure in a firm-fitting pocket or even attached through a covalent bond, such as the heme ring associated with cytochrome C molecules which is bonded through thioether linkages with adjacent cysteine residues (Figure 1.16). Cofactors, by contrast, may be bound only transiently to proteins during periods of activity. Enzymes often require cofactors to act as donors or acceptors of chemical groups that are added to or cleaved from a substrate molecule. Some common cofactors are ATP, ascorbic acid, coenzyme A, NAD, NADP, FAD, FMN, and biotin. Sometimes, the enzyme cofactor also is an energy source for the catalytic reaction, as in the case of ATP dependent reactions.

Frequently, metal ions are associated with the prosthetic group or cofactor. Heme rings usually contain a chelated iron atom. Occasionally, however, these metals are merely bound within folded polypeptide regions with no additional organic constituents required. Many metal ions are known to participate in enzymatic activity. One or more of the ions of Na, K, Ca, Zn, Cu, Mg, Mn, as well as Co and Mo are often required by enzymes to maintain activity.

Prosthetic groups and cofactors, whether organic or metallic, may be removed from a protein to create an inactive *apo* protein or enzyme. Loss of these groups may occur through environmental changes, such as removing metal ions from solution or adding denaturants to unfold

protein structure. In many cases, simply re-introducing the needed group into the surrounding medium can restore full activity.

In addition to small organic molecules or metal ions, proteins may have other components tightly associated with them. Nucleoproteins, for instance, contain noncovalently bound DNA or RNA, as in some of the structural proteins of viruses. Lipoproteins contain associated lipids or fatty acids and may also carry cholesterol, as in the high-density and low-density lipoproteins in serum.

During modification or conjugation reactions, prosthetic groups and other associated molecules may be lost or damaged. Metal ions temporarily may be removed by the inclusion of a chelating agent added to maintain sulfhydryl stability during coupling through the —SH groups of a protein. To restore activity after conjugation, it is necessary to remove the chelator and add the required metal salts. Other changes to the prosthetic carriers may not be so easily corrected. For instance, heme-containing molecules are sensitive to the presence of agents that can form a coordination complex with or modify the oxidation state of the chelated metal ion. Some reagent systems may permanently inactivate the heme-containing protein.

Thus, loss of activity can occur not only through changes to the amino acid constituents of a protein, but through prosthetic group or cofactor loss or damage as well. Most of these potential difficulties can be overcome through careful selection of the reaction conditions and through knowledge of the cofactor dependencies that are critical to the activity of the protein being modified.

Post-translational modifications to protein structure are covalent changes that occur as the result of controlled enzymatic reactions or due to chemical reactions not under enzymatic regulation. One of the most common cellular modifications performed on proteins after ribosomal synthesis is glycosylation. Proteins newly synthesized on ribosomes, may be transported to the Golgi apparatus where specific glycosyl transferases catalyze the coupling of carbohydrate residues to the polypeptide chains. Glycoproteins and mucoproteins are formed by the coupling of polysaccharides through *o*-glycosidic linkages to serine, threonine, or hydroxylysine and through *N*-glycosidic linkages with the amide side chain group of asparagine.

The structure of most glycoprotein carbohydrate is branched with the sugars mannose, *N*-acetyl glucosamine, sialic acid, galactose, and L-fucose being prevalent. Asparagine-linked polysaccharides are well characterized and are known to be constructed of a core unit consisting of three mannose residues and two *N*-acetyl glucosamine (GlcNAc) residues. The GlcNAc residues are bound to the Asp side chain amide nitrogen through a β1 linkage (Kornfield and Kornfield, 1985). The three mannose groups then usually form the first branch point in the oligosaccharide chain (Chapter 1, Section 2).

The content by weight of carbohydrate in glycoproteins may vary from only a few percent to over 50 percent in some proteins in mucous secretions. Although the function of the polysaccharide in most glycoproteins is unknown, in some cases it may provide hydrophilicity, recognition, and points of noncovalent interaction with other proteins through lectin-like affinity binding.

The presence of carbohydrate on protein or peptide molecules can provide important points of attachment for modification or conjugation reactions. Coupling exclusively through polysaccharide chains often can direct the reaction away from active centers or critical points in the polypeptide chain, thus preserving activity. Polysaccharides can be specifically targeted on glycoproteins through mild sodium periodate oxidation. Periodate cleaves adjacent hydroxyl groups in sugar residues to create highly reactive aldehyde functionalities (Chapter 1, Section 4.4).

The level of periodate addition can be adjusted to selectively cleave only certain sugars in the polysaccharide chain. For instance, a concentration of 1 mM sodium periodate at temperatures less than 4°C specifically oxidizes sialic acid residues to contain aldehydes, leaving all other monosaccharides untouched. Increasing the concentration to 10 mM and doing the reaction at room temperature, however, will cause oxidation of other sugars in the carbohydrate chain, including galactose and mannose. The generated aldehydes then can be used in coupling reactions with amine or hydrazide containing molecules to form covalent linkages. Amines can react with formyl groups under reductive amination conditions using a suitable reducing agent such as sodium cyanoborohydride. The result of this reaction is a stable secondary amine linkage (Chapter 2, Section 5.3). Alternatively, hydrazides spontaneously react with aldehydes to form hydrazone linkages, although the addition of a reducing agent increases the efficiency of the reaction (Chapter 2, Section 5.1).

Another form of post-translational modification that may add carbohydrate to a polypeptide is non-enzymatic glycation. This reaction occurs between the reducing ends of sugar molecules and the amino groups of proteins and peptides. See Section 2.1 in this chapter for further details and the reaction sequence behind this modification.

Protecting the Native Conformation and Activity of Proteins

The goal of most protein modification or conjugation procedures is to create a stable product with good retention of the native state and activity. Ideally, any derivatization should result in a protein that performs exactly as it would in its unmodified form, but with the added functionality imparted by whatever is conjugated to it. Thus, an antibody molecule tagged with a fluorophore should retain its ability to bind to antigen and also have the added functionality of fluorescence.

One of the best ways to ensure retention of activity in protein molecules is to avoid doing chemistry at the active center. The active center is that portion of the protein where ligand, antigen, or substrate binding occurs. In simpler terms, the active center (or active site) is that part that has specific interaction with another substance (Means and Feeney, 1971). For the preparation of enzyme derivatives, it is important to protect the site of catalysis where conversion of substrate to product happens. For instance, when working with antibody molecules, it is crucial to stay away from the two antigen binding sites.

The best chemical procedures avoid the active site by selecting functional groups away from that area or by protecting the site through the incorporation of additives. In some cases, the inclusion of substrates, cofactors, ligands, inhibitors, or antigens in the modification reaction will protect the active site. Addition of the appropriate substance can bind the active site and mask it from modification by crosslinking agents. In enzyme derivatization procedures, this is often just a matter of adding a reversible inhibitor or substrate analog. For instance, when working with alkaline phosphatase merely doing the reaction in phosphate buffer protects the active center from chemical modification, since phosphate ions bind in the catalytic site. With trypsin, the incorporation of benzamidine similarly masks and protects the active site.

However, protecting the antigen binding sites on an antibody molecule by using this method is often more difficult. Inclusion of antigen to mask the binding sites is effective in blocking these areas, but it also may cause irreversible crosslinking of the antigen to the antibody. This is especially true when the antigen is a peptide or a protein having the same chemical functionalities as the antibody. Any modification reactions that are directed at the antibody may modify the antigen as well. Therefore, only use this method if the antigen is lacking in the chemical

targets that are going to be used on the antibody. For instance, if the polysaccharide chains on the antibody are targeted for modification, then using a protein antigen that does not contain carbohydrate to block the antigen binding sites may work well.

An equally effective method of protecting the activity of a protein is by using site-directed reactions that result in modifications away from the active center. In some cases, specific functionalities are known to be present only at restricted sites within the three-dimensional structure of a protein. If these functionalities are not present close to the active site, then using them exclusively for modification reactions should assure good retention of activity. For instance, sulfhydryl groups or carbohydrate chains are often present in limited quantity and in specific regions on a protein. Selecting reagent systems that target these groups assures derivatization only at restricted sites within the protein molecule, thus potentially avoiding the active center.

Surprisingly, the goal of some protein crosslinking schemes is to somewhat alter the native presentation of the conjugate. This is especially true in hapten–carrier conjugation as used for immunogen or vaccine preparation. In this case, the main objective is to modify the environment of the hapten to create an immunological response *in vivo*. A hapten is usually a small molecule that is not able to generate an immune response on its own, but can react with the products of such a response once generated. Most often these products are antibodies having binding specificity for the hapten.

The complexities involved in achieving a successful conjugation strategy are best illustrated in the problems and concerns dealing with hapten–carrier conjugation. In order to produce the initial immune response to a small molecule, the hapten is typically coupled to a larger protein that can generate a response on its own. In simple terms, the larger carrier protein confers immunogenicity to the smaller hapten. The native presentation of the hapten is altered toward the immune system, thus creating the immune response.

The site of attachment of the hapten to the carrier and the nature of the crosslinker are both important to the specificity of the resultant antibodies generated against it. For proper recognition, the hapten must be coupled to the carrier with the appropriate orientation. For an antibody subsequently to recognize the free hapten without the attached carrier, the hapten–carrier conjugate must present the hapten in an exposed and accessible form. Optimal orientation is often achieved by directing the crosslinking reaction to specific sites on the hapten molecule. With peptide haptens, this is typically done by attaching a terminal cysteine residue during synthesis. This provides a free thiol group on one end of the peptide for conjugation to the carrier. Crosslinking through this group provides hapten attachment only at one end, therefore ensuring consistent orientation.

In hapten–carrier conjugation, the goal is not to maintain the native state or stability of the carrier, but to present the hapten in the best possible way to the immune system. In reaching this goal, the choice of conjugation chemistry may control the resultant titer, affinity, and specificity of the antibodies generated against the hapten. It may be important in some cases to choose a crosslinking agent containing a spacer arm long enough to present the antigen in an unrestricted fashion. It also may be important to control the density of the hapten on the surface of the carrier. Too little hapten substitution may result in little or no response. A hapten density too high actually may cause immunological suppression and decrease the response. In addition, the crosslinker itself may generate an undesired immune response. Fortunately, for the majority of hapten–carrier conjugation problems, a few main crosslinking techniques provide a workable compromise to solving all these concerns and ultimately generating an effective immune response (Chapter 19).

Oxidation of Amino Acids in Proteins and Peptides

The modification of amino acids in proteins and peptides by oxidative processes plays a major role in the development of disease and in aging (Halliwell and Gutteridge, 1989, 1990; Kim *et al*., 1985; Tabor and Richardson, 1987; Stadtman, 1992). Tissue damage through free radical oxidation is known to cause various cancers, neurological degenerative conditions, pulmonary problems, inflammation, cardiovascular disease, and a host of other problems. Oxidation of protein structures can alter activity, inhibit normal protein interactions, modify amino acid side chains, cleave peptide bonds, and even cause crosslinks to form between proteins.

Due to their abundance in cells relative to other biological molecules, proteins are one of the primary targets of oxidation *in vivo*. However, sometimes oxidation reactions involving proteins and peptides are thought of solely as the creation of disulfides from thiols on cysteine residues. This is certainly an important form of oxidation that can affect protein structure and function or even cause problems relevant to bioconjugation reactions. The presence of an accessible free thiol on a protein in an aqueous solution can be highly unstable to rapid oxidation unless precautions are taken to prevent disulfide formation. Dissolved oxygen and other potentially catalytic components, such as certain metal salts, quickly can result in disulfides being formed within a protein or between different protein molecules.

From a broader perspective, protein oxidation can result in covalent modification at many sites other than just at cysteine thiols. The earliest reports on protein oxidation date from the first decade of the twentieth century, but it took many more years to characterize these reactions and their products (Dakin, 1906).

The significance of protein oxidation became paramount with the advent of recombinant protein biologics used as human therapeutics. Careful characterization of protein stability is essential to maintaining the efficacy of protein pharmaceuticals. If even a single side chain amino acid residue becomes oxidized, then a protein therapeutic may not have the same activity *in vivo* as the unmodified protein.

Oxidation of proteins can result from exposure to oxidative species from many sources: reactive oxygen intermediates caused by metabolic reactions within cells (mitochondrial electron transport function and certain enzymes, such as oxidases, peroxidases, and P-450 enzymes), from the by-products of oxidative stress reactions in cells (Sayre *et al*., 2001), or through the presence of strongly oxidizing compounds within a solution—all of these can contribute to selective damage or modification to protein structures. Some examples of chemical agents that oxidatively can modify proteins include hydrogen peroxide (H_2O_2) and other peroxy compounds, such as perborate and peroxycarbonate; hydroperoxyl radical ($HO_2^{\bullet}$); superoxide anion ($O_2^{\bullet -}$); singlet oxygen ($^1O_2$); hydroxyl radical ($^{\bullet}OH$), periodate (IO_4^-); metal salts in the presence of oxygen species, such as those of iron (Fe^{3+} and Fe^{2+}) and copper (Cu^{2+}); ozone (O_3); peroxynitrite ($ONOO^-$); Hypobromous acid (HOBr); hypochlorous acid (HOCl); performic acid (HC(O)OOH); trichloromethylperoxyl radical ($CCl_3OO^{\bullet}$); under the right conditions metal-chelating compounds, such as porphyrins, texaphyrins, and FeBABE; and gamma radiation and UV light. For additional information see Winterbourn and Kettle (2000); Baynes and Thorpe (2000); Greenacre and Ischiropoulos (2001); Halliwell and Gutteridge (1989, 1990); Stadtman (1992).

Singlet oxygen ($^1O_2$) differs from the predominant oxygen molecule in that O_2 is in the ground state or triplet state and its outer two unshared electrons have parallel spins (sometimes designated $^3O_2$), which is nearly unreactive toward other molecules, while singlet oxygen

has increased energy and has its outer electrons transformed into an opposite spin orientation, which is highly reactive. Superoxide ($O_2^{\bullet -}$) is different from singlet oxygen in that it is a reduced form of oxygen having an extra unpaired electron, called a radical. The presence of the radical makes superoxide extremely reactive and highly damaging to proteins and other biological molecules.

Singlet oxygen and superoxide, in addition to their modifying effects on proteins, also are important reactive oxygen species in biological applications, as they are intermediates used in some detection methods and in photodynamic therapy (PDT) for the treatment of cancer. One of the more common compounds used in PDT is Photofrin, which is a mixture of oligomers consisting of ether and ester linkages that combine up to eight porphyrin groups (Misawa *et al.*, 2005). The generation of reactive oxygen species takes place by irradiation with 630 nm wavelength laser light, which also penetrates the skin effectively during therapy. Photoactivation of the Photofrin molecule causes radical initiation to form porphyrin-excited states. Transfer of electrons from the porphyrin groups to molecular oxygen then generates the highly reactive singlet oxygen species. Subsequent radical reactions also can form superoxide and hydroxyl radicals, all of which severely damage tissue in the region of the tumor and ultimately cause cancer cell death.

Another compound used to generate reactive oxygen species for PDT is texaphyrin, which contains a metal-chelating ring structure resembling a porphyrin group (Figure 1.17). Typically, a gadolinium atom is chelated in the texaphyrin center and this complex becomes both a photosensitizing agent and a magnetic resonance imaging (MRI) contrast agent to better visualize tumor locations for irradiation therapy (Donnelly *et al.*, 2004).

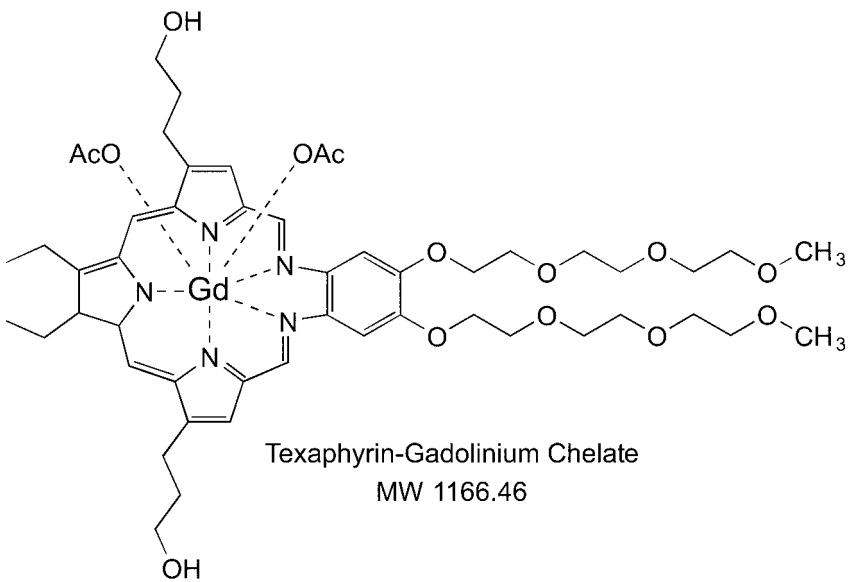


Figure 1.17 The texaphyrin–gadolinium chelate structure used as a photosensitizer and MRI contrast agent in the detection and treatment of cancer.

The reactive oxygen species involved with protein oxidation can be generally categorized according to their relative reactivity as follows:

$$HO^{\bullet}, HO_2^{\bullet} > O_2^{\bullet -} > ROOH, H_2O_2 > {}^1O_2, ClO^-, BrO^- > O_2$$

Thus, radicals are the most reactive and destructive of protein structure, followed by peroxy derivatives, singlet oxygen, and other oxygen compounds. The oxidative reactivity of some of these oxygen species is so high that just contact of the pure compound with paper or cotton fabrics can cause combustion (e.g., superoxide).

In vitro studies of protein oxidation indicate that virtually all proteins and peptides are susceptible to damage by the radicals: $^{\bullet}OH$ and $O_2^{\bullet -}$ Analysis of protein modification products after oxidation indicates the presence of altered molecular weight (either fragmentation or oligomerization), altered net charge, tryptophan destruction, and the formation of tyrosine dimers (Davies, 1987). Even in the presence of very low concentrations of oxidants (nM), SDS polyacrylamide gel electrophoresis of proteins can indicate multiple bands of higher and lower molecular weight due to oxidative damage.

Transition metals in solution can catalyze the formation of reactive oxygen species that are particularly damaging to proteins and other biomolecules. In a series of reactions, reduced transition metals, such as Fe^{2+} and Cu^{1+}, can be oxidized by oxygen to produce superoxide and ultimately undergo a Fenton reaction to create hydroxyl radicals (Kim *et al*., 1985). Transition metal-chelating groups can accelerate this reaction, as demonstrated in the process of hydroxyl radical footprinting of protein interactions using EDTA chelates of iron (see discussion on the reagent FeBABE in Chapter 28, Section 4).

$$\text{Production of superoxide: } Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$$

$$\text{Production of hydrogen peroxide: } 2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$\text{Production of hydroxyl radical: } Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

The potential sites of oxidation within a protein molecule include the peptide backbone and the side chain amino acid groups. Hydrogen atom abstraction at the α-carbon of an amino acid chain can occur upon reaction with an oxidative species to form a radical intermediate. Subsequent reaction can result in peptide bond cleavage and fragmentation of the protein structure, often forming carboxylic acids or carbonyls (aldehydes or ketones). This is the basic mechanism of fragmentation caused by the bifunctional chelating reagent FeBABE. When used in the presence of H_2O_2 and ascorbic acid, polypeptides will fragment in the neighborhood of interacting proteins.

Amino acid side chains can undergo oxidation through hydrogen abstraction, elimination, or by addition reactions. In the presence of oxygen, aliphatic amino acids usually experience oxidation to a peroxy intermediate, which causes either hydrogen atom abstraction or an elimination reaction resulting in the formation of carbonyls, hydroxyls, or other peroxides (Requena *et al*., 2001) (Figure 1.18). Aromatic amino acids typically undergo addition reactions following exposure to strong oxidants. An example of this type of reaction is the nitrosation of tyrosine groups in the presence of a peroxynitrite ($ONOO^-$) to create *o*-nitrotyrosine (see Figure 1.19).

After exposure to an oxidant, the potential types of oxidation products in proteins and peptides can be extensive (Stadtman and Levine, 2000). Cysteine and methionine undergo a variety of sulfur oxidation reactions to yield cysteine disulfides, methionine sulfoxide, methionine

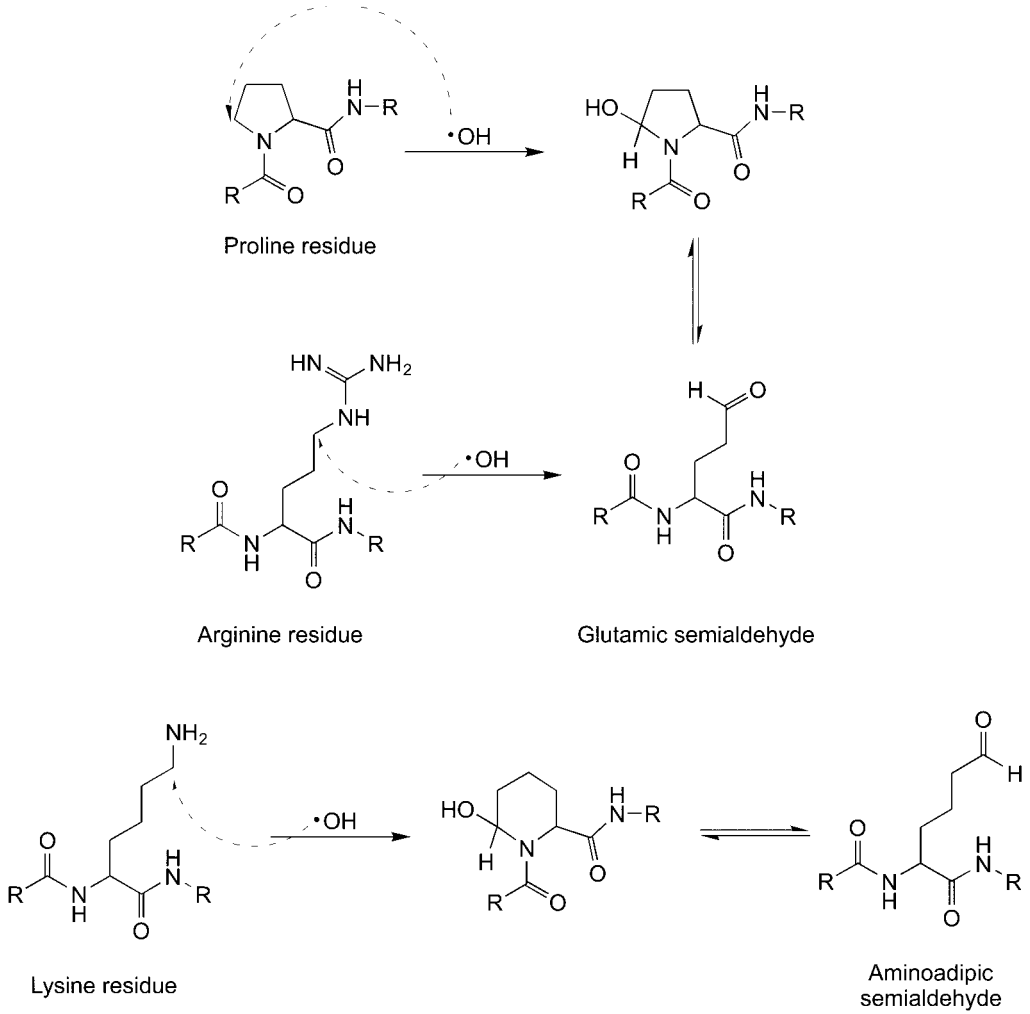


Figure 1.18 Reaction of proline, arginine, and lysine residues with hydroxyl radical results in oxidation of side-chain structures that form carbonyls. Both arginine and proline oxidation result in the same product.

sulfone, and sulfonate products (e.g., cysteic acid) (Figure 1.20). Oxidation with performic acid can be used purposely to convert methionine and cysteine in peptides and proteins to more stable products prior to acid hydrolysis and amino acid analysis. Cysteine and methionine are perhaps the most sensitive amino acids to oxidation, and for this reason, they are an early indicator of oxidative damage to proteins.

Tyrosine also is easily modified through addition reactions due to the ring activating nature of its phenolic group. Using oxidants, tyrosine's ring can be chlorinated, iodinated, undergo nitrosation or hydroxylation, and even form tyrosine–tyrosine crosslinks. The last product can be formed purposely by use of a peroxidase in the presence of hydrogen peroxide, and this type of reaction has been studied extensively in the manufacture of phenolic polymer resins

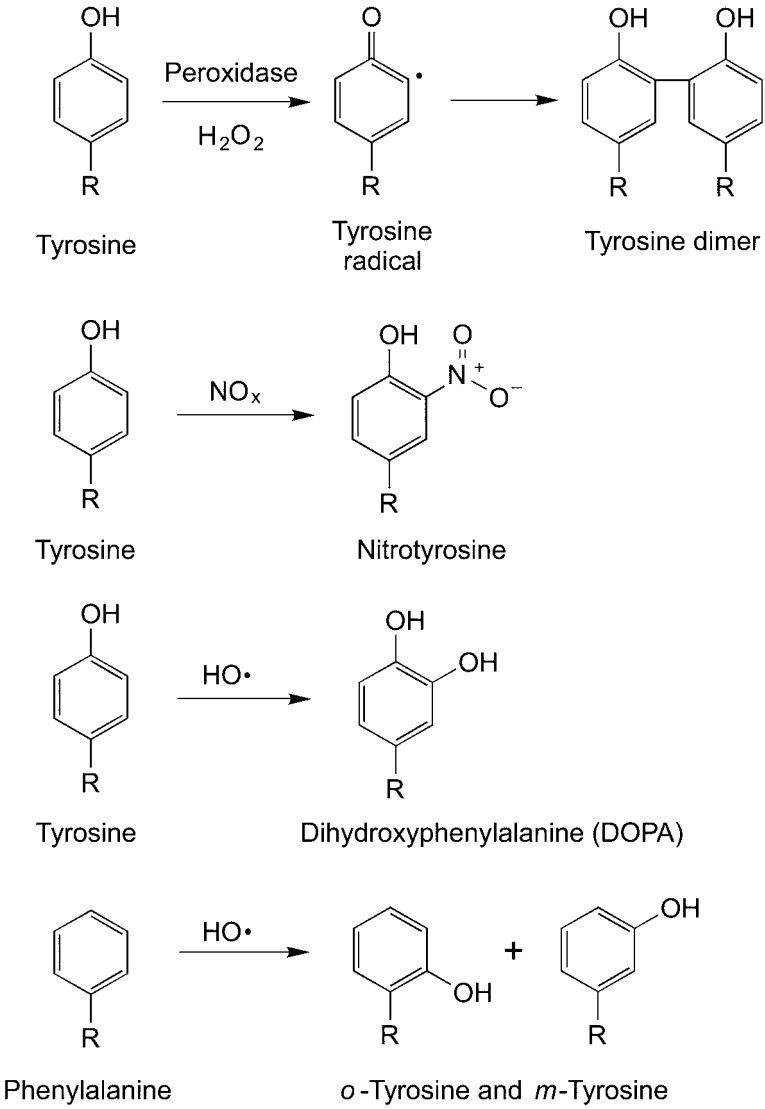


Figure 1.19 Tyrosine and phenylalanine residues can undergo oxidation to modify their phenyl side-chain groups. Tyrosine can form covalent dimers that link two side chains together via a radical reaction. Both tyrosine and phenylalanine can be modified by oxidation to add oxygen-containing groups directly to their aromatic ring.

(Dordick, 1991). In addition, Fancy *et al.* (1996) as well as Fancy and Kodadek (1997, 1998) have applied the oxidation of tyrosine to form dityrosine to the study of protein–protein interactions using nickel-chelated 6 × His tagged fusion proteins in oxidative environments.

Nitrogen-containing side chains in amino acids can be altered by oxidation forming chloramines or even become deaminated. The result is often the formation of carbonyls (e.g., aldehydes) and hydroxyls. Lee, S *et al.* (2006) found that Fe-EDTA-mediated oxidation of human

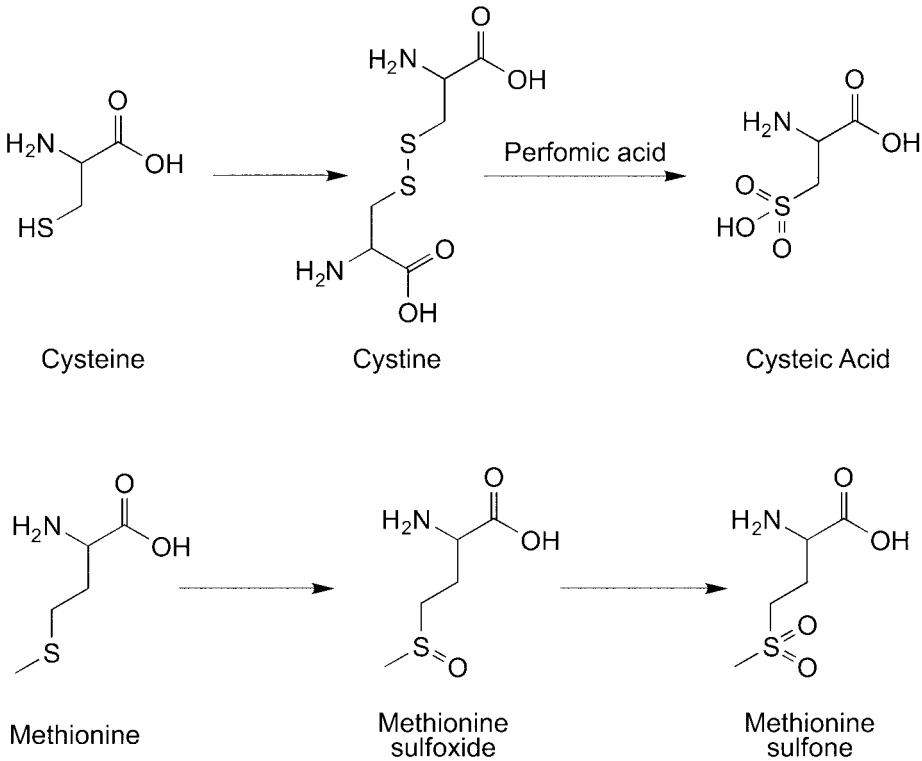


Figure 1.20 Cysteine and methionine are highly susceptible to oxidation reactions. Cysteine thiols can form disulfide linkages with other cysteine groups or be oxidized to cysteic acid. Methionine is oxidized very easily to the sulfoxide or sulfone products.

serum albumin resulted in extensive aldehyde and ketone formation from modification of lysine, arginine, histidine, proline, threonine, and aspartic and glutamic acids. Some groups will oxidize and convert to another amino acid altogether. For instance, histidine can be converted to asparagine, proline to hydroxyproline, and tyrosine changed to dihydroxyphenylalanine (DOPA) through oxidation reactions.

It is obvious that the oxidation of protein molecules can have detrimental effects on protein structure and function. However, there are some unique methods in bioconjugation wherein controlled and purposeful oxidation is done to study protein–protein interactions (Chapter 28, Section 4).

Unfortunately, there are no universal methods to detect all types of protein oxidation, because the products formed can be so diverse in nature. However, some forms of protein oxidation can be assayed using chemical modification (Davies *et al.*, 1999; Shacter, 2000). In particular, the formation of carbonyl groups on proteins can be targeted using the reagent 2,4-dinitrophenylhydrazine (DNPH). This compound reacts with aldehydes to form 2,4-dinitrophenylhydrazone derivatives, which create chromogenic modifications that can be detected at high sensitivity in microplate assays or Western blot analysis (Buss *et al.*, 1997; Winterbourn *et al.*, 1999).

In addition, a method involving mass spec analysis to determine carbonyl formation as a result of protein oxidation was developed using a novel mass tag. The carbonyl-specific Element-Coded Affinity Mass Tag (O-ECAT) can covalently couple to aldehyde or ketone oxidation sites using an aminoxy group to form an oxime (Lee *et al.*, 2006; see also Chapter 16, Section 2). The ECAT mass tag consists of a bifunctional metal-chelating group that coordinates a lanthanide metal ion of specific mass. Proteins that have been oxidized to contain carbonyls can be labeled with this reagent and the exact sites of modification determined by analyzing the mass spec signature of the labeled peptides after proteolysis.

Solvent Accessibility of Functional Targets in Proteins

Proteins are highly complex, folded polypeptide chains consisting of at least 20 different amino acids that are strung together in unique sequences, which relate to structure and function. Particular amino acids in proteins may be further modified post-translationally to contain a wide variety of covalent modifications normally found in native proteins. The way in which a peptide chain is wrapped and folded governs each amino acid's relative exposure to the outside environment, but post-translational modifications also can obscure the protein surface from easy access to the solvent environment.

Amino acid side chains are the primary effectors of the three-dimensional structure of a protein, because their properties vary depending on the presence of charged groups, uncharged polar components, aliphatic chains, aromatic rings, and groups able to form hydrogen bonds with other amino acid residues. The relative hydrophilicity or hydrophobicity of an amino acid side chain is a major factor in determining whether the group will be found on the surface of a globular protein or buried within its globular structure.

However, just considering the individual properties of each amino acid type is not enough to determine its accessibility to the surrounding aqueous environment. There have been many attempts at developing analytical models with predictive value for determining buried or surface accessible amino acids in a folded polypeptide chain. These studies have concluded fractional assignments for each residue that relate to its accessible surface area (ASA) or its solvent exposed area (SEA).

In most cases, there are general trends that emerge from theoretical studies in which hydrophilic amino acids are more likely to be found on the surface of a protein and hydrophobic amino acids are more likely to be inside its three-dimensional structure, but we already knew this intuitively so this conclusion is not surprising. However, a real-life study of the positions of amino acids in proteins whose structures are known is more revealing. The data for Figure 1.21 was calculated from 55 proteins in the Brookhaven database by Bordo and Argos (1991), and the graph was derived from the analysis as presented by the Jena Image Library of Biological Macromolecules (http://www.imb-jena.de/IMAGE_AA.html). Although most of these structures were determined using crystallographic means and thus the proteins are "frozen" in a single structural state, the results are revealing as to how often particular amino acids are accessible to the surrounding solvent.

Three levels of SEA are presented in the graph for each amino acid, which corresponds to areas in $Å^2$ accessible to the solvent environment: greater than 30 $Å^2$ for highly accessible amino acids, between 10 and 30 $Å^2$ for medium accessibility, and less than 10 $Å^2$ for those residues that are relatively not accessible to the solvent. Only the SEA for each amino acid of >30 $Å^2$ is shown in the plotted data. The graph shows that the polar amino acids such as serine, threonine,

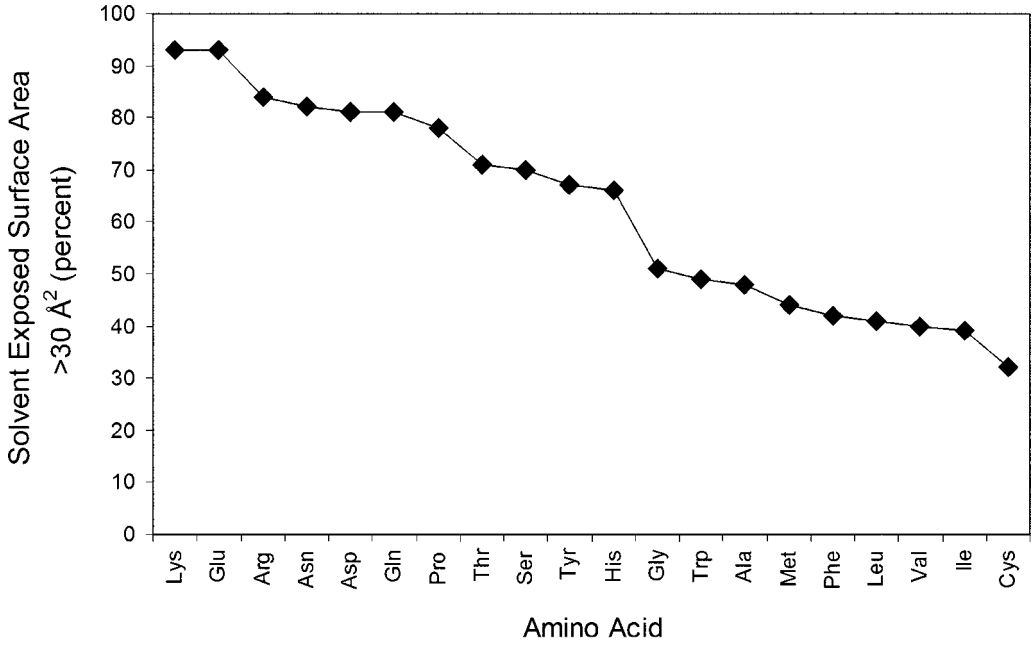


Figure 1.21 Comparison of the solvent exposed surface area of amino acids in proteins. Data are plotted as a percentage of each amino acid in a protein having greater than a 30 Å^2 exposure to the aqueous environment. Charged and polar amino acids are seen to have the most solvent exposure, while uncharged, aromatic, or aliphatic amino acids have the least exposure.

asparagine, glutamine, and tyrosine often have large areas accessible to the solvent, as do the charged amino acids aspartic acid, glutamic acid, lysine, histidine, and arginine. Surprisingly, proline also falls in the highly accessible group, which is not as expected, because it doesn't carry a charge, nor is it a highly polar amino acid. However, proline does have a unique characteristic that may explain its appearance on the surface of proteins: it cannot freely rotate about its imino group as other amino acids in a peptide chain can do at amide bonds. This effect results in a kink in the polymer (called a beta-turn), and these sharp turns in a peptide backbone probably occur most often near the surface. Thus proline is found to be frequently accessible to the solvent environment despite its hydrophobic nature.

The non-polar amino acids glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, and cysteine have lower exposure to the solvent environment than charged or polar residues. However, the frequency at which these groups are found to have an SEA of greater than 30 Å^2 is much higher than one would expect based solely upon consideration of their hydrophobicity. In fact, nearly 30–50 percent of the time non-polar amino acids in a protein can be found at the surface.

At the two extremes, lysine is observed as the amino acid most accessible on the surface of proteins while cysteine is the least exposed amino acid. The inaccessibility of cysteine probably stems from the fact that disulfides are typically buried within the polypeptide structure of proteins, whether they are intrachain or interchain in nature, and proteins rarely contain many reduced cysteine thiols.

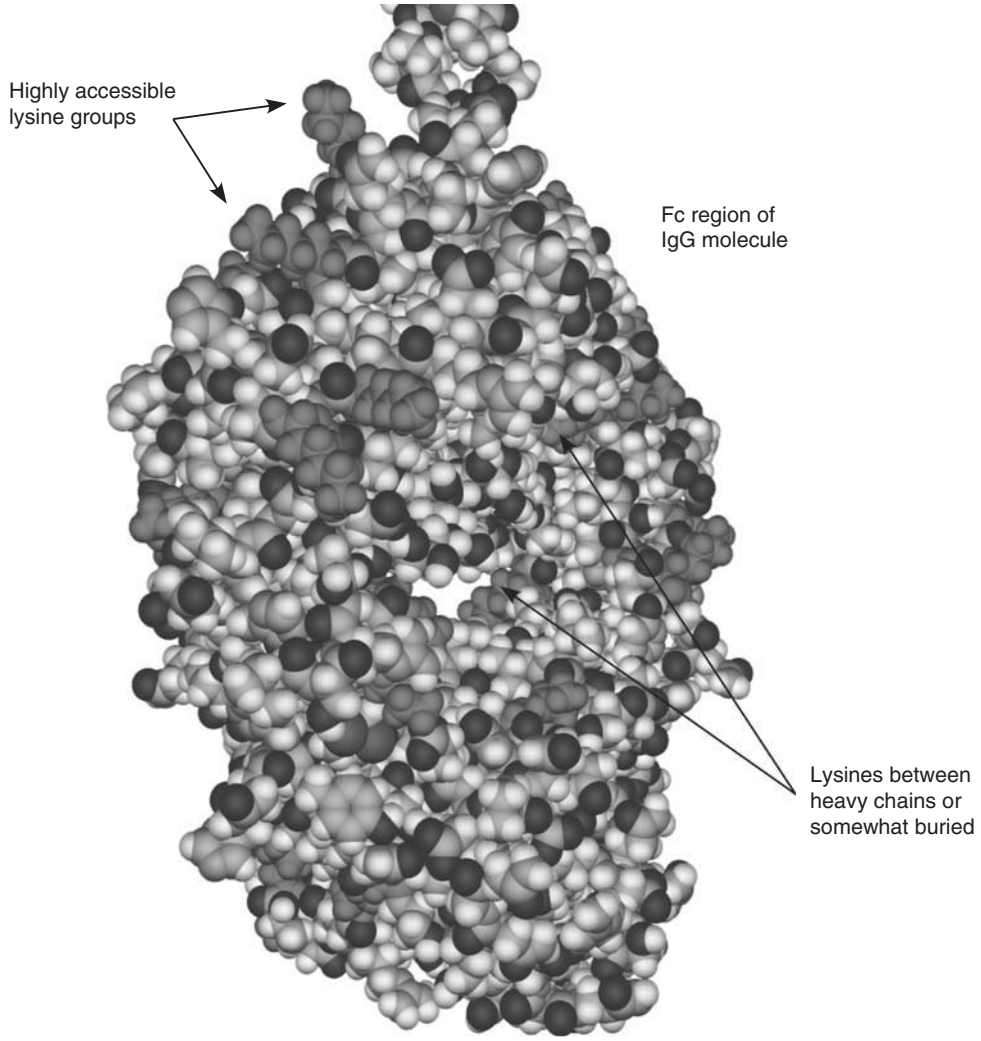


Figure 1.22 The solvent accessibility of lysine residues in the Fc region of an antibody is illustrated by highlighting the lysine groups in solid gray. Some lysine ε-amine groups are extremely accessible to conjugation, while others are only partially exposed, making them difficult to modify in bioconjugation reactions.

It is clear from this data that proteins have complex hydrophilic and hydrophobic regions on their surfaces that determine their potential interactions, binding sites, and active centers. For bioconjugation purposes, targeting of an amino acid even with a high SEA for modification or crosslinking may not result in every residue being modified that is theoretically present in a protein based only on knowledge of its amino acid composition. Even when coupling to very polar or charged groups, such as lysine, there are varying degrees of accessibility to a given reagent, because of the complex folding of the polypeptide chains at the protein surface.

Figure 1.22 shows the globular structure of an immunoglobulin (IgG) Fc region to illustrate this point. In this space-filling model, the lysine residues are highlighted in solid gray to easily

show their locations within the two polypeptides of the heavy chains. Notice that some of the ε-amino groups at the ends of the side chains are protruding far out into the solvent and are therefore highly accessible for modification. Some of these groups, however, are less exposed even though they are still near the surface, and a few lysines are seen to be between the heavy chain regions where it would be difficult to modify them due to crowding.

Figure 1.64 in this chapter provides data to validate this effect. The reaction of the thiolating reagent SATA (*N*-succinimidyl *S*-acetylthioacetate) with IgG resulted in only a percentage of the available lysines being modified. As the molar ratio of SATA to IgG was increased, the yield of lysine modification actually became lower. This result can be explained by the relative accessibility of each lysine in the immunoglobulin structure. Some residues are easily accessible and they get modified with high yield even with low molar ratios of SATA-to-IgG. As the molar ratio is increased, it gets more difficult to modify those lysines that are less accessible to the solvent environment or are partially obscured by another polypeptide chain. Thus, the solvent accessibility of particular amino acids is a major factor in whether they can be effectively targeted and modified with a given bioconjugate reagent.

1.2. Protein Crosslinking Methods

The crosslinking of two proteins using a simple homobifunctional reagent (Section 2.2) potentially can result in a broad range of conjugates being produced (Avrameas, 1969). The reagent initially may react with either one of the proteins, forming an active intermediate. This activated protein may then form crosslinks with the other protein or with another molecule of the same protein. The activated protein also may react intramolecularly with other functionalities on part of its own polypeptide chain. Other crosslinking molecules may continue to react with these conjugated species to form various mixed products, including severely polymerized proteins that may fall out of solution (Figure 1.23).

The problems of indeterminate conjugation products are amplified in single-step reaction procedures using homobifunctional reagents (Chapter 4). Single-step procedures involve the addition of all reagents at the same time to the reaction mixture. This technique provides the least control over the crosslinking process and invariably leads to a multitude of products, only a small percentage of which represent the desired or optimal conjugate. Excessive conjugation may cause the formation of insoluble complexes that consist of very high-molecular-weight polymers. For example, one-step glutaraldehyde conjugation of antibodies and enzymes (Chapter 20, Section 1.2) often results in significant oligomers and precipitated conjugates. To overcome this shortcoming, multi-step reaction procedures have been developed using both homobifunctional and heterobifunctional reagents (Chapter 5). Controlled, multi-step conjugation protocols alleviate the polymerization problem and form relatively low molecular weight, soluble antibody–enzyme complexes (Chapter 20, Section 1.1).

In two-step protocols, one of the proteins to be conjugated is reacted or "activated" with a crosslinking agent and excess reagent and by-products are removed. In the second stage, the activated protein is mixed with the other protein or molecule to be conjugated, and the final conjugation process occurs (Figure 1.24).

The use of homobifunctional reagents in two-step protocols still creates many of the problems associated with single-step procedures, because the first protein can crosslink and

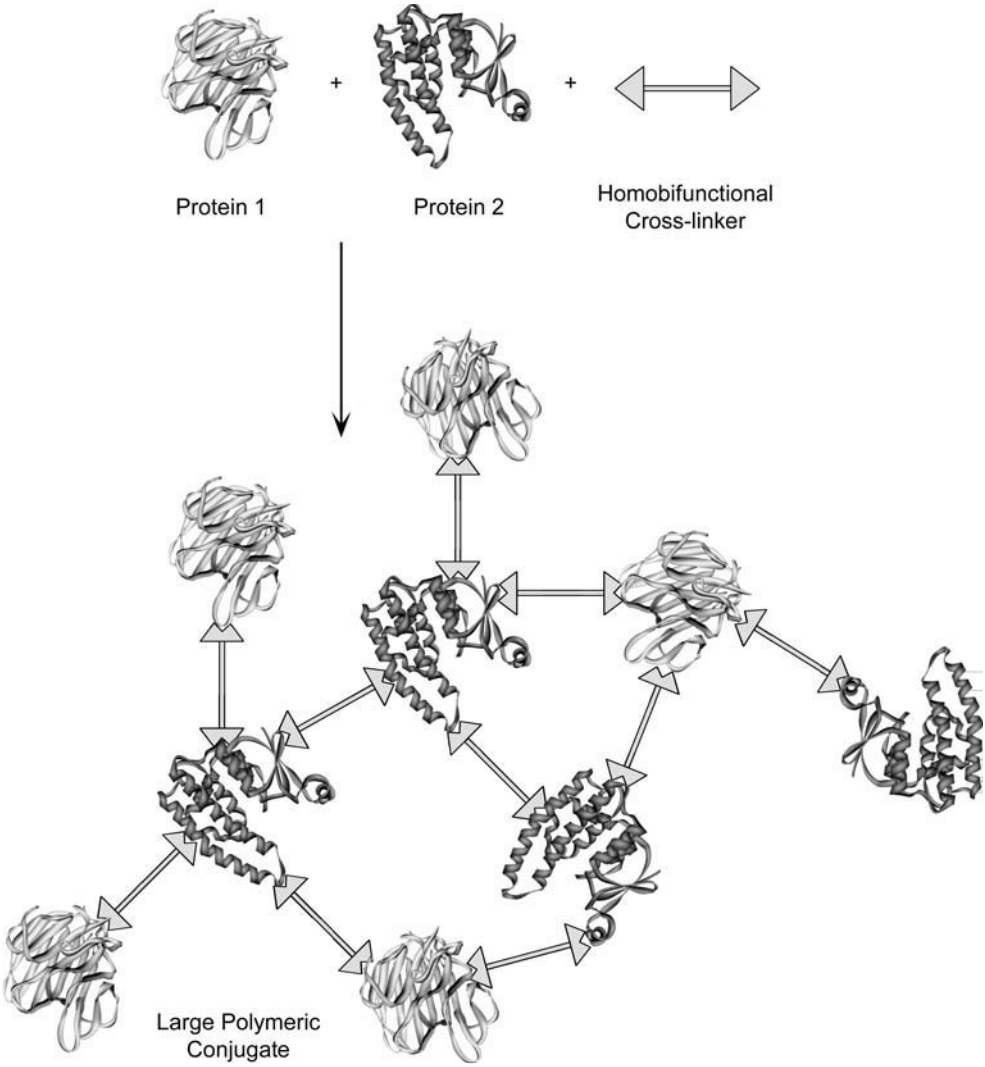


Figure 1.23 Protein crosslinking reactions done using homobifunctional reagents can result in large polymeric complexes of multiple sizes and indefinite structure.

polymerize with itself long before the second protein is added. Homobifunctional reagents by definition have the same reactive group on both ends of the crosslinking molecule. Since the protein to be activated has target functionalities on every molecule that can couple with the reactive groups on the crosslinker, both ends of the reagent potentially can react. This inherent potential to uncontrollably polymerize unfortunately is characteristic of all homobifunctional reagents, even in multi-step protocols.

The greatest degree of control in crosslinking procedures is afforded using heterobifunctional reagents (Chapter 5). Since a heterobifunctional crosslinker has different reactive groups on either end of the molecule, each side can be directed specifically toward different functional

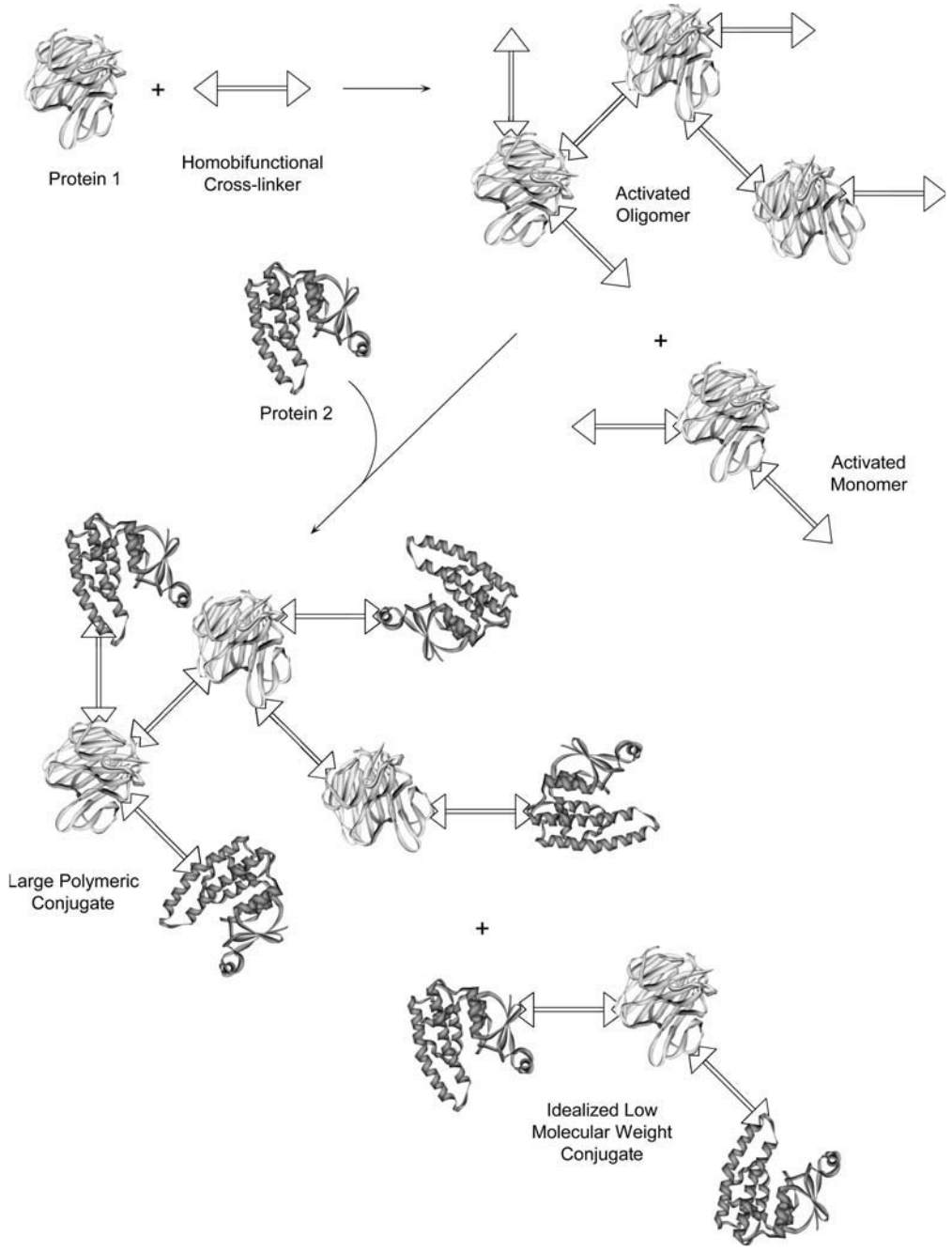


Figure 1.24 A two-step protocol using a homobifunctional crosslinking agent offers more control than single-step methods, but still may result in oligomer formation.

groups on proteins. Using a multi-step conjugation protocol with a heterobifunctional reagent can allow one macromolecule to be activated, excess crosslinker removed, and then a second macromolecule added to induce the final linkage. Directed conjugation will occur as long as the first protein that is activated doesn't have groups able to couple with the second end of the crosslinker, whereas the second molecule does possess the correct functionalities.

Occasionally, the second protein doesn't naturally have the target groups necessary to couple with the second end of the crosslinker. In such cases, a specific functionality usually can be created to make the conjugation successful (Chapter 1, Section 4). In such three-step systems, the first protein is activated with the heterobifunctional reagent and purified away from excess crosslinker. The second protein is then modified to contain the specific target groups required for the second stage of the conjugation. Finally, in step three, the two modified proteins are mixed to cause the coupling reaction to happen (Figure 1.25).

Two- and three-step protocols using heterobifunctional crosslinkers often are designed around amine-reactive and sulfhydryl-reactive chemical reactions. Many of these reagents utilize NHS esters on one end for coupling to amine groups on the first protein and maleimide groups on the other end that can react with sulfhydryls on the second protein. The NHS ester end is reacted with the first protein to be conjugated, forming an activated intermediate containing reactive maleimide groups. Fortunately, the maleimide end of such crosslinkers is relatively stable to degradation, thus the activated protein can be isolated without loss of sulfhydryl coupling ability. Additionally, if the second protein does not contain indigenous sulfhydryls, these can be created by an abundance of methods (Chapter 1, Section 4.1). After mixing the maleimide-activated protein with the sulfhydryl-containing protein, conjugation can occur only in one direction.

Control of the products of conjugation increases as the protocols progress from single-step to multi-step reactions. Likewise, control of the chemistry of conjugation increases as the reagent systems evolve from simple homobifunctional to site-directed heterobifunctional. It may appear to be a paradox, but often as the method of conjugation gets more complex the result is less potential for side reactions and therefore fewer products being formed. Therefore, multi-step processes using advanced heterobifunctional reagents are the best combination to assure that the protein conjugate formed is indeed the one desired.

2. Modification of Sugars, Polysaccharides, and Glycoconjugates

The basic units of food energy for cells and living organisms consist of polysaccharides or simple sugars, principally glucose and its derivatives. Biological molecules themselves often contain carbohydrate or are made exclusively of such components. Complex carbohydrate "trees" frequently project off the surface of cells, providing specific points of attachment or sites of recognition. Lipids and proteins that contain these components may possess them to give identity or partial hydrophilicity to their parent structures.

Many of the macromolecules that are the subject of modification or conjugation reactions contain significant proportions of carbohydrate. Reactions can be designed to target directly these polysaccharide portions, either selectively modifying them with small, detectable compounds or using them as conjugation bridges to couple with other macromolecules. The reactivity of carbohydrate molecules in such derivatizations is an important factor in the success of many bioconjugate techniques.

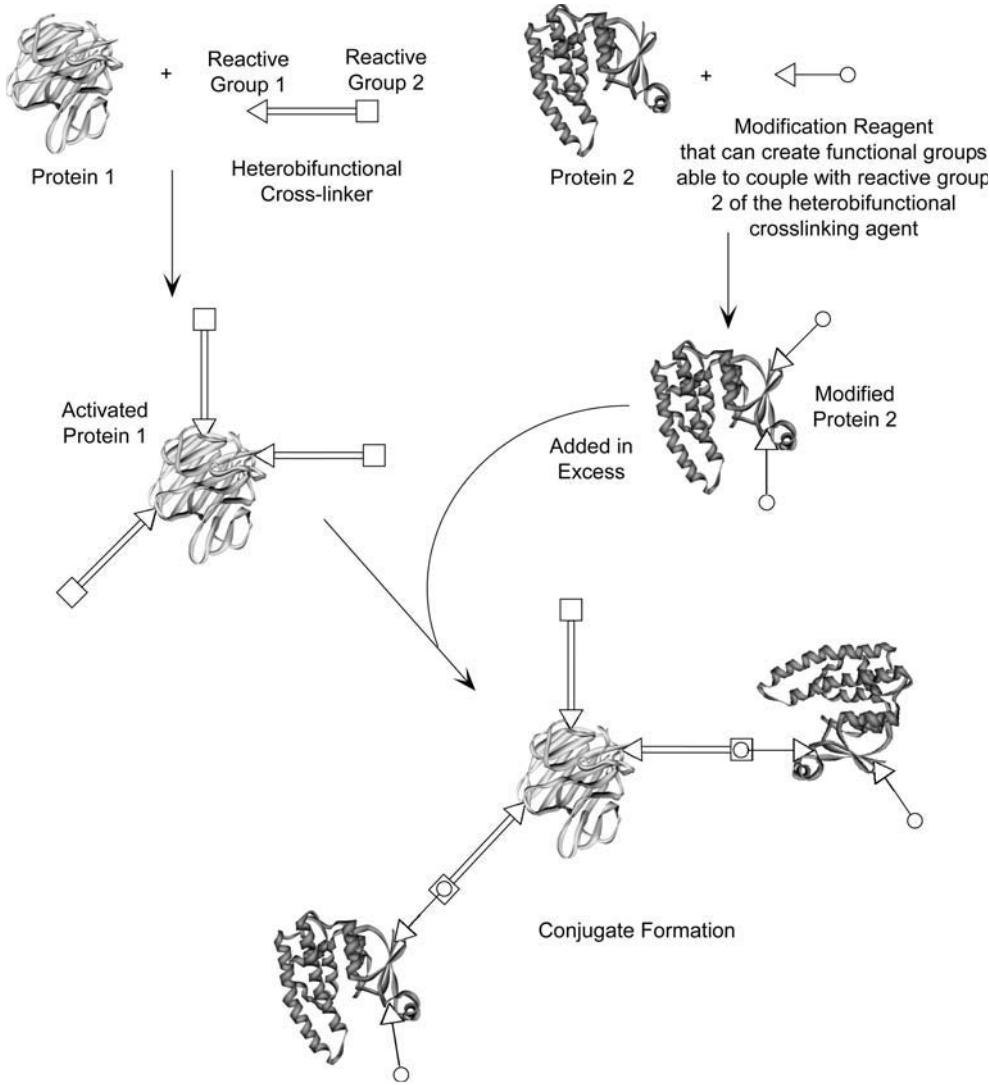


Figure 1.25 Heterobifunctional crosslinking agents used in multi-step protocols result in the best control over the products formed.

This section describes the basic chemical attributes of carbohydrate molecules. Principal sites of reactivity on carbohydrates are discussed with the aim of developing a rational approach to using them in modification and conjugation procedures.

2.1. Carbohydrate Structure and Functionality

Carbohydrates are characterized by the presence of polyhydroxylic aldehyde or polyhydroxylic ketone structures or polymers made of such units. Sugars and polysaccharides have definite

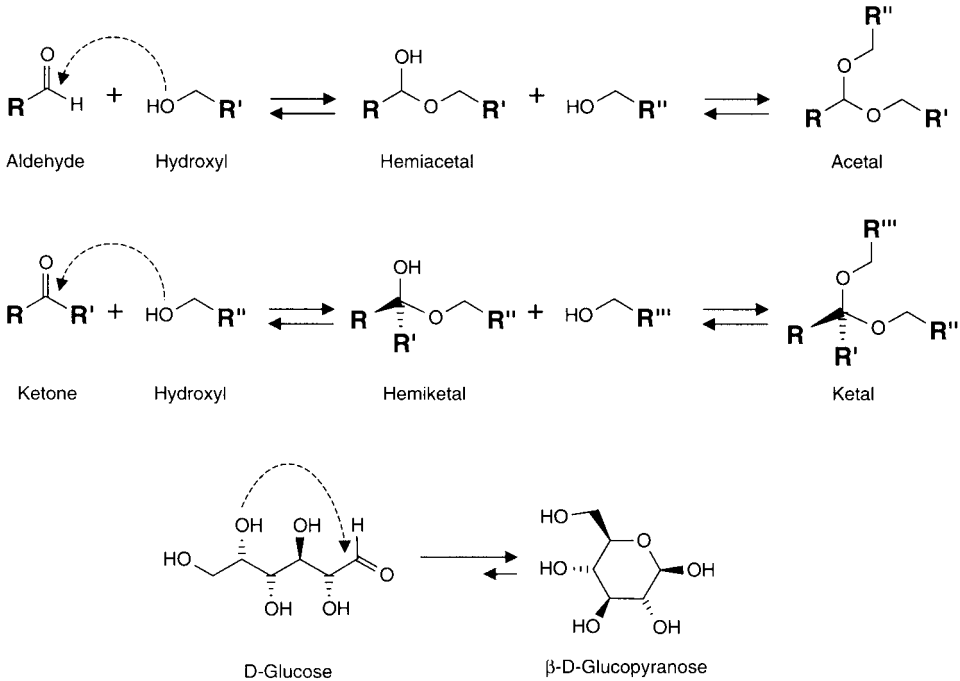


Figure 1.26 Carbonyl groups and hydroxyls may react to form acetal or ketal products. Sugars naturally undergo these reactions to form ring structures in aqueous solution.

three-dimensional structures that are important for many biological functions. They are hydrophilic and thus easily accessible to aqueous reaction mediums. The chemistry of bioconjugation using carbohydrate molecules begins with an understanding of the building blocks of polysaccharide molecules.

Basic Sugar Structure

The simplest carbohydrate, called a monosaccharide, is composed of a structure that cannot be hydrolyzed to simpler polyhydroxylic compounds. A disaccharide is a carbohydrate that contains two of these basic units, and a polysaccharide contains many polyhydroxylic monomers.

A monosaccharide that contains an aldehyde group is called an aldose, and one that contains a ketone group is a ketose. Monosaccharides are further classified by the number of carbon atoms they contain. Thus, a five-carbon sugar is known as a pentose and a six-carbon sugar, a hexose. All monosaccharides containing accessible aldehyde or ketone functionalities are reducing sugars—that is they are able to reduce Fehling's or Tollen's reagent.

The aldehyde or ketone group of monosaccharides can undergo an intramolecular reaction with one of its own hydroxyl groups to form a cyclic, hemiacetal, or hemiketal structure, respectively (Figure 1.26). In aqueous solutions, this cyclic structure actually predominates. The open-chain aldehyde or ketone form of monosaccharides is in equilibrium with the cyclic form, but the open structure exists less than 0.5 percent of the time in aqueous environments. It is the

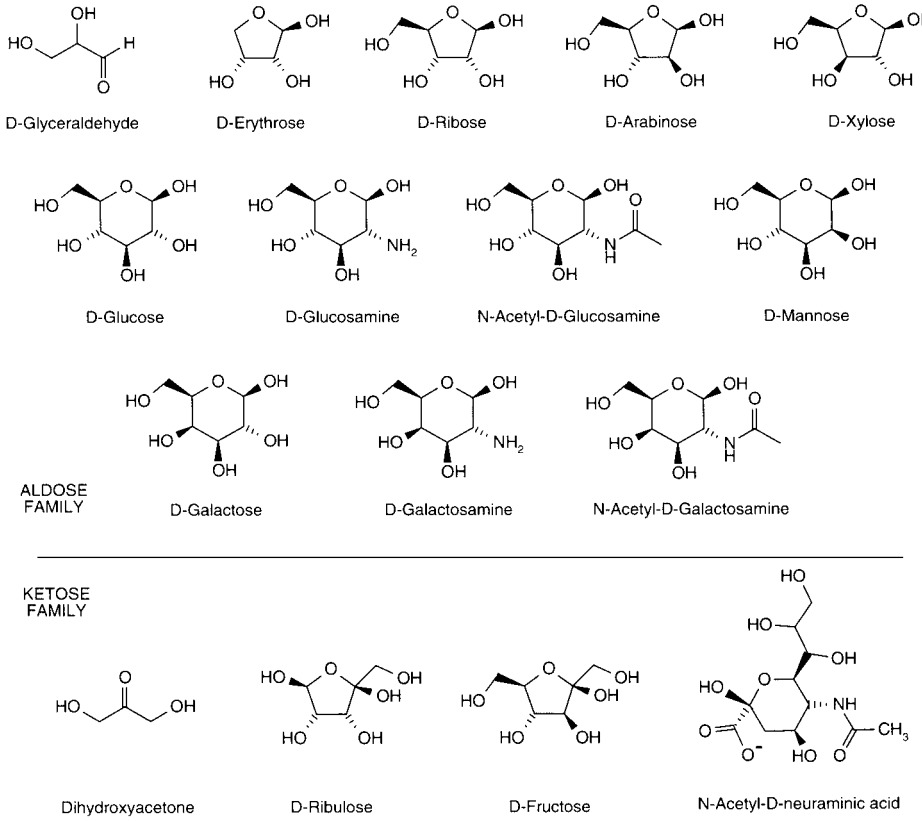


Figure 1.27 Common monosaccharides of the aldose and ketose families found in biological molecules.

open form that reduces Fehling's or Tollen's reagent. However, due to this predominance of the cyclic structure of monosaccharides, they do not have the capability of reacting with bisulfite or Schiff's reagent, as do normal unblocked aldehydes and ketones. Thus, the carbonyl functionalities of sugars have reduced reactivity, because of hemiacetal and hemiketal formation.

Figure 1.27 shows the structures of some of the most common monosaccharide molecules: D-glyceraldehyde, D-erythrose, D-ribose, D-arabinose, D-xylose, D-glucose, D-glucosamine, *N*-acetyl-D-glucosamine, D-mannose, D-galactose, D-galactosamine, *N*-acetyl-D-galactosamine of the aldose family and dihydroxyacetone, D-ribulose, D-fructose, D-*N*-acetylneuraminic acid of the ketose family. Formation of the cyclic structure of each of these sugars can result in one of two stereoisomers, designated α and β, depending on the orientation of the aldehyde group or ketone group during hemiacetal formation. For aldoses, the α form is drawn in the standard Haworth projection with the No. 1 carbon hydroxyl pointing down. For ketoses, the α form consists of the No. 2 carbon hydroxyl pointing down. All the common monosaccharide structures shown in Figure 1.27 are in the β-stereoisomer form.

Since in aqueous solutions the cyclic form of monosaccharides is in equilibrium with their corresponding open forms, the α and β structures continually interconvert. At equilibrium, one form usually predominates. For instance, glucose dissolved in water consists of about a 2:1

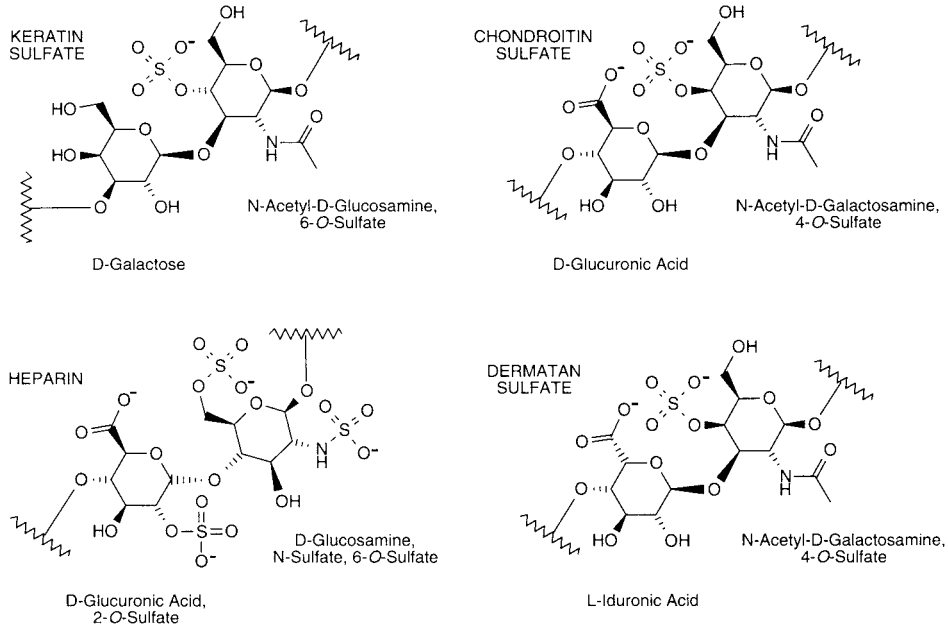


Figure 1.28 Common sulfonated polysaccharides of biological origin.

ratio of β-D-glucose to α-D-glucose. Although their chemical constituents are identical, the biochemical properties between the α and β forms can be quite different. Monosaccharides linked together to form disaccharides and polysaccharides cannot continue to interconvert and are therefore frozen in the α or β forms. Changing one monosaccharide in a complex carbohydrate to its opposite stereoisomer form can produce radical structural changes in the polysaccharide chain and significantly alter its biochemical properties.

Sugar Functional Groups

Monosaccharide functional groups consist of either a ketone or an aldehyde, several hydroxyls, and the possibility of amine, carboxylate, sulfate, or phosphate groups as additional constituents. Amine-containing sugars may possess a free primary amine, but often are modified to the *N*-acetyl derivative, such as the *N*-acetyl glucosamine residue of chitin. Sulfate-containing monosaccharides frequently are found in certain mucopolysaccharides, including chondroitin sulfate, dermatan sulfate, heparin sulfate, and keratin sulfate (Figure 1.28). Carboxylate-containing sugars include sialic acid as well as many aldonic, uronic, oxoaldonic, and ascorbic acid derivatives (Figure 1.29). Phosphate-containing monosaccharides are almost exclusively created in metabolic processes involving energy utilization, such as in the production of glucose-1-phosphate formed during glycogen breakdown and glucose-6-phosphate produced during glycolysis. Perhaps the most common phosphate sugar derivative, however, is the 5′-phosphate of D-ribose or D-2-deoxyribose found as a repeating component of RNA and DNA, respectively.

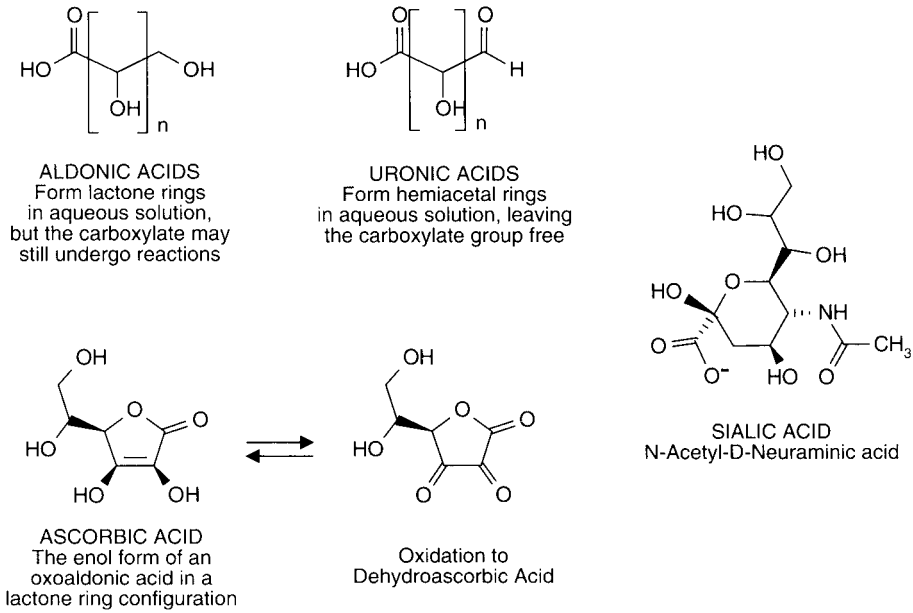


Figure 1.29 Monosaccharides containing carboxylate groups. Sialic acid often is found at the terminal residues of polysaccharides within glycoproteins.

Modification and conjugation reactions can be designed to target many of these functionalities. Sugar hydroxyl groups, for example, may be derivatized by acylating or alkylating reagents, similar to the principal reactions of primary amines (Section 1). However, acylation of a hydroxyl group usually creates an unstable ester derivative that is subject to hydrolysis in aqueous solution. An exception to this is acylation by a carbonylating reagent such as carbonyldiimidazole (CDI) (Chapter 2, Section 4.2) or *N*,*N*′-disuccinimidyl carbonate (DSC) (Chapter 2, Section 4.3), which can produce stable carbamate linkages after subsequent conjugation with an amine-containing molecule. By contrast, alkylating reagents, such as alkyl halogen compounds (Chapter 2, Section 4.6) typically form more stable ether bonds after reaction with hydroxyls. Figure 1.30 shows the reactions associated with alkylation and acylation of hydroxyl residues.

Carbohydrates-containing hydroxyl groups on adjacent carbon atoms may be treated with sodium periodate (Section 4.4, this chapter) to cleave the associated diol carbon–carbon bond and oxidize the hydroxyls to reactive formyl groups (Bobbitt, 1956). Modulating the concentration of sodium periodate can direct this oxidation to exclusively modify sialic acid groups (using 1 mM concentration at temperatures $<4°C$) or to convert all available diols to aldehydes (using 10 mM or greater concentrations at room temperature). Specific monosaccharide residues may be targeted with selective sugar oxidases to generate similar aldehyde functions only at discrete points within a complex polysaccharide structure (Section 4.4, this chapter) (Avigad *et al.*, 1962; Gahmberg, 1978). The creation of formyl groups in this manner may be done on purified polysaccharide molecules, as in the case of soluble dextrans (Chapter 25, Section 2.1), or may be selectively performed on carbohydrate constituents of glycoproteins and other glycoconjugates. Once formed, aldehyde groups may be covalently coupled with

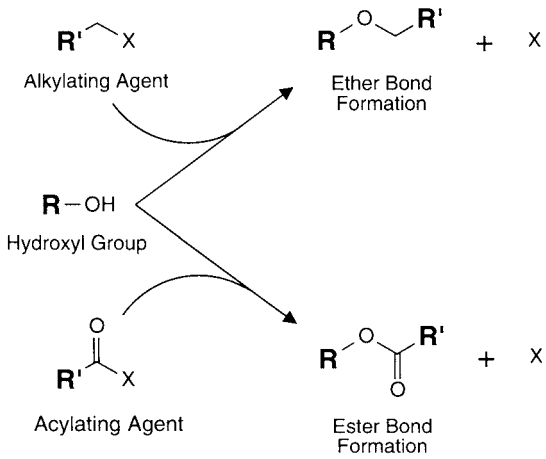


Figure 1.30 Hydroxyl groups within sugar residues may undergo alkylation or acylation reactions, forming ether or ester linkages.

amine-containing molecules by reductive amination using sodium cyanoborohydride (Chapter 3, Section 4) (Dottavio-Martin and Ravel, 1978; Cabacungan *et al.*, 1982).

The native reducing ends of carbohydrates also may be conjugated to amine-containing molecules by reductive amination. The reaction, however, typically is less efficient than using periodate-created aldehydes, since the open structure is in low concentration in aqueous solutions compared to the cyclic hemiacetal form. The reaction is usually allowed to continue for a week or more to reach good yields of coupling. Proteins may be modified to contain carbohydrate using this procedure (Gray, 1974; Baues and Gray, 1977; Schwartz and Gray, 1977; Gray, 1978). See Section 4.6 of this chapter for a more complete discussion of methods for the introduction of saccharide or glycan groups into proteins or other molecules.

The reducing ends of oligosaccharides can be modified with β-(*p*-aminophenyl)ethylamine to yield terminal arylamine derivatives (Jeffrey *et al.*, 1975; Zopf *et al.*, 1978a, b). The aromatic amines then can be diazotized for coupling to active-hydrogen-containing molecules, such as the tyrosine phenolic residues in proteins (Zopf *et al.*, 1978b). Alternatively, the arylamines may be transformed into isothiocyanate derivatives for coupling to amine-containing molecules, such as proteins (Smith, D. F *et al.*, 1978). The aromatic amine also may be used to conjugate the modified oligosaccharide directly with amine-reactive crosslinking agents or probes.

Another potential reaction of created or native aldehyde groups on carbohydrates is with hydrazide functionalities to form hydrazone linkages. Hydrazide-containing probes or crosslinking reagents may be conjugated with periodate-oxidized polysaccharides or with the reducing ends of sugars. The hydrazone bonds may be reduced with sodium cyanoborohydride to more stable linkages (Chapter 2, Section 5.1). The reduction step is recommended for long-term stability of crosslinked molecules. An example of this modification strategy is the use of biotin–hydrazide (Chapter 11, Section 3) to label specifically glycoproteins at their carbohydrate locations.

Reducing sugars can be detected by reaction with phenylhydrazine to yield a hydrazone product, except the result of the reaction is not what one might imagine giving the structure of aldoses and ketoses. Glucose, for example, can react with phenylhydrazine to yield the anticipated

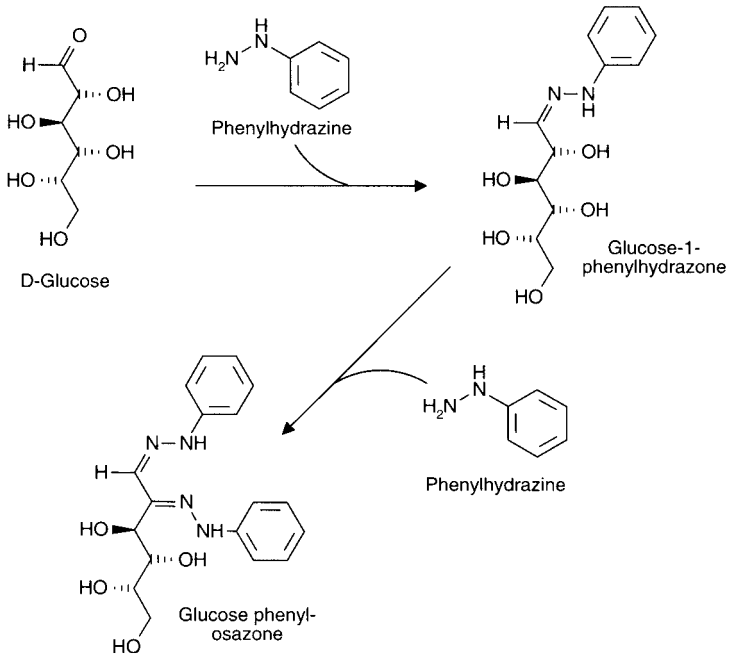


Figure 1.31 Phenylhydrazine can react with aldehyde or ketone groups within carbohydrates to give detectable products.

1-phenylhydrazone derivative. In an excess of phenylhydrazine, however, the reaction continues to yield a 1,2-phenylhydrazone product, called an osazone, with concomitant production of aniline and ammonia (Figure 1.31). Exactly how the No. 2 hydroxyl group gets oxidized to react with another molecule of phenylhydrazine is not entirely clear, but probably proceeds through an enol intermediate. This reaction is typical of all α-hydroxy aldehydes and α-hydroxy ketones, not just those occurring in carbohydrate molecules. Thus, glucose, mannose, and fructose all yield the same osazone product upon reaction with phenylhydrazine, since the stereochemical differences about carbons 1 and 2 are eliminated. Reversal of the phenylhydrazone linkage with an excess of benzaldehyde yields an osone, a 1-aldehyde-2-keto-derivative of the sugar. Many simple hydrazide-containing reagents probably are capable of forming similar 1,2-hydrazone derivatives with reducing sugars, provided their size does not cause steric difficulties.

Polysaccharides, glycoproteins, and other glycoconjugates therefore may be specifically labeled on their carbohydrate portions by creating aldehyde functionalities and subsequently derivatizing them with another molecule containing an amine or a hydrazide group. This route of derivatization is probably the most common way of modifying carbohydrates.

The hydroxyl residues of polysaccharides also may be activated by certain compounds that form intermediate reactive derivatives containing good leaving groups for nucleophilic substitution. Reaction of these activated hydroxyls with nucleophiles such as amines results in stable covalent bonds between the carbohydrate and the amine-containing molecule. Activating agents that can be employed for this purpose include CDI (Chapter 2, Section 4.2 and Chapter 3, Section 3), certain chloroformate derivatives (Chapter 1, Section 4.3), tresyl- and tosyl-chloride, cyanogen

bromide, divinylsulfone, cyanuric chloride (Chapter 25, Section 1.1), disuccinimidyl carbonate (Chapter 4, Section 1.7), and various *bis*-epoxide compounds (Chapter 2, Section 1.7). Such activation steps are frequently done in nonaqueous solutions (i.e., dry dioxane, acetone, DMF, or DMSO) to prevent hydrolysis of the active species. While many pure polysaccharides can tolerate these organic environments, many biological glycoconjugates cannot. Thus, these methods are suitable for activating pure polysaccharides such as dextran, cellulose, agarose, and other carbohydrates, but are not appropriate for modifying sugar residues on glycoproteins. Many of these hydroxyl-activating reagents also can be used to activate polysaccharide chromatography supports and other hydroxyl containing synthetic polymers such as polyethylene glycol or hydroxylic particles (Chapter 14). For a complete treatment of polysaccharide chromatographic support activation through hydroxyl groups, see Hermanson *et al.* (1992). For a description of the activation of soluble polysaccharides and synthetic polymers, see Chapter 25.

While the hydroxyl groups on carbohydrate molecules are nucleophilic in aqueous solution, they are approximately equal to water in relative nucleophilicity. Since the majority of reactive functionalities on bioconjugation reagents are dependent upon nucleophilic reactions to initiate covalent bond formation, specific hydroxyl group modification is usually not possible in aqueous solution—especially with other biomolecules displaying stronger nucleophilic groups as well (e.g., amines and thiols). In many instances, hydrolysis of the active groups on crosslinking reagents occurs faster than hydroxyl group modification, due to the relative high abundance of water molecules compared to the amount of carbohydrate hydroxyls present. In some cases, even if modification does occur, the resultant bond may be unstable. For instance, NHS esters (Chapter 2, Section 1.4) can react with hydroxyls to form ester linkages, which are themselves unstable to hydrolysis.

Anhydrides, such as acetic anhydride (Sections 4.2 and 5.1, this chapter), may react with carbohydrate hydroxyls even in aqueous environments to form acyl derivatives. The reaction, however, is reversible by incubation with hydroxylamine at pH 10–11.

Epoxide-containing reagents, such as the homobifunctional 1,4-(butanediol) diglycidyl ether (Chapter 4, Section 7.1), can react with polysaccharide hydroxyl groups to form stable ether bonds. Bis-epoxy compounds have been used to couple sugars and polysaccharides to insoluble matrices for affinity chromatography (Sundberg and Porath, 1974). The reaction of epoxides, however, is not specific for hydroxyl groups and will cross-react with amine and sulfhydryl functionalities, if present.

Hydroxyl groups on carbohydrates may be modified with chloroacetic acid to produce a carboxylate functionality for further conjugation purposes (Plotz and Rifai, 1982). In addition, indigenous carboxylate groups, such as those in sialic acid residues and aldonic or uronic acid containing polysaccharides, may be targeted for modification using typical carboxylate modification reactions (Chapter 2, Section 3). However, when these polysaccharides are part of macromolecules containing other carboxylic acid groups such as glycoproteins, the targeting will not be specific for the carbohydrate alone. Pure polysaccharides containing carboxylate groups may be coupled to amine-containing molecules by use of the carbodiimide reaction (Chapter 3, Section 1). The carboxylate is activated to an *o*-acylisourea intermediate, which is in turn attacked by the amine compound. The result is the formation of a stable amide linkage with loss of one molecule of isourea.

Carbohydrate molecules containing amine groups, such as D-glucosamine, easily may be conjugated to other macromolecules using a number of amine reactive chemical reactions and crosslinkers (Chapter 2, Section 1 and Chapter 3). Some polysaccharides containing acetylated

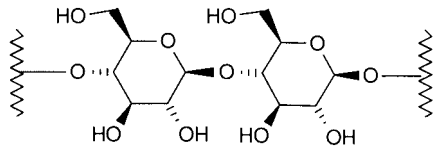
amine residues, such as chitin which contains *N*-acetyl-glucosamine, may be deacetylated under alkaline conditions (Jeanloz, 1963) to free the amines (forming chitosan in this case).

Amine functionalities also may be created on polysaccharides (Section 4.3, this chapter). The reducing ends of carbohydrate molecules (or generated aldehydes) may be reacted with small diamine compounds to yield short alkylamine spacers that can be used for subsequent conjugation reactions. Hydrazide groups may be similarly created using *bis*-hydrazide compounds (Sections 4.5 and 4.6, this chapter).

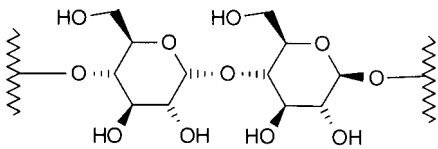
Phosphate containing carbohydrates that are stable, such as the 5′-phosphate of the ribose derivatives of oligonucleotides, may be targeted for modification using a carbodiimide-facilitated reaction (Section 4.3, this chapter). The water-soluble carbodiimide EDC (1-ethyl-3-(3-di methylaminopropyl)carbodiimide) can react with the phosphate groups to form highly reactive phospho-ester intermediates. These intermediates can react with amine or hydrazide-containing molecules to form stable phosphoramidate bonds.

Polysaccharide and Glycoconjugate Structure

Aldose monosaccharide units are frequently bound together through the No. 1 carbon hydroxyl group of one sugar to another sugar's No. 4 or 6 hydroxyl group, forming a complete acetal linkage. Two monosaccharides coupled in this fashion are termed a disaccharide. Numerous monosaccharides bound together to form a chain are called a polysaccharide. The most abundant polysaccharides in nature, starch and cellulose, consist of glucose bound together in α-1,4 and β-1,4, and to a lesser extent, α-1,6, acetal linkages (Figure 1.32). While the hemiacetal, cyclic structure of individual sugars shows some reversibility under equilibrium conditions, the acetal linkage between two monosaccharides is quite stable, only hydrolyzing under severe pH extremes.



Cellobiose repeating unit
of cellulose;
D-Glucose joined in
β-1,4-linkages



Maltose repeating unit
of starch (amylose);
D-Glucose joined in
α-1,4-linkages

Figure 1.32 The repeating units of cellulose and starch, two of the most common polysaccharides in nature.

Similarly, ketose sugars participate in polysaccharide formation by reaction of their anomeric carbon with a hydroxyl of another monosaccharide to create a ketal linkage. The acetal and ketal bonds within polysaccharides are termed *o*-glycosidic linkages.

Hemiacetal hydroxyl groups of carbohydrate molecules also may be coupled to amine-containing molecules to form *N*-glycosidic linkages, such as those in nucleic acids and oligonucleotides.

Polysaccharides may or may not have reducing power, depending on the way they are linked together and whether the terminal, potentially reducing end is available. The structure of simple disaccharides can illustrate this point. Of the most common disaccharides, sucrose and lactose, sucrose is a non-reducing sugar since β-D-fructose is linked through its reducing C-2 hydroxyl, and lactose remains a reducing sugar, since the terminal glucose is linked to β-D-galactose through its C-4 hydroxyl, leaving its reducing end free (Figure 1.33).

Polysaccharide synthesis is under enzymatic control, but does not occur from a template as in protein synthesis. For this reason, each molecule of a particular polysaccharide will have its own unique molecular weight. The molecular weight of a carbohydrate polymer is usually expressed as an average. Starch or cellulose chains, for example, may vary by several hundred thousand in their molecular weights between individual molecules. For an excellent review of carbohydrate chemistry, see Binkley (1988).

Due to their polyhydroxylic structures, all carbohydrates are polar and will possess associated water molecules in aqueous solution, but they may not be fully water-soluble. Large polysaccharides such as cellulose form intricate matrices created from extensive hydrogen bonding. Neighboring monosaccharide units hydrogen bond within the same chain, while neighboring polymers form interchain hydrogen bonds between hydroxyls. The three-dimensional structure of a carbohydrate to a large extent is determined by these hydrogen bonds—sometimes resulting in sheeted or helical structures, as in the triple helix of agarose polysaccharide chains. Water will be intimately associated in this internal arrangement, but the overall multi-polymer structure often is too large to allow for complete water solubility. For a review, see Preis (1980).

Polysaccharide solubility in aqueous solutions usually is dependent on polymer size and its allied three-dimensional structure. Even water-insoluble carbohydrates may be solubilized by controlled hydrolysis of *o*-glycosidic linkages to create smaller polysaccharide molecules. Thus, cellulose may be solubilized by heating in an alkaline solution until the polymers are broken up sufficiently to reduce their average molecular weight. Many such soluble forms of common polysaccharides are available commercially.

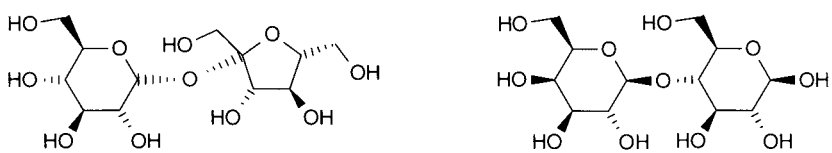


Figure 1.33 Comparison of a reducing and a non-reducing disaccharide.

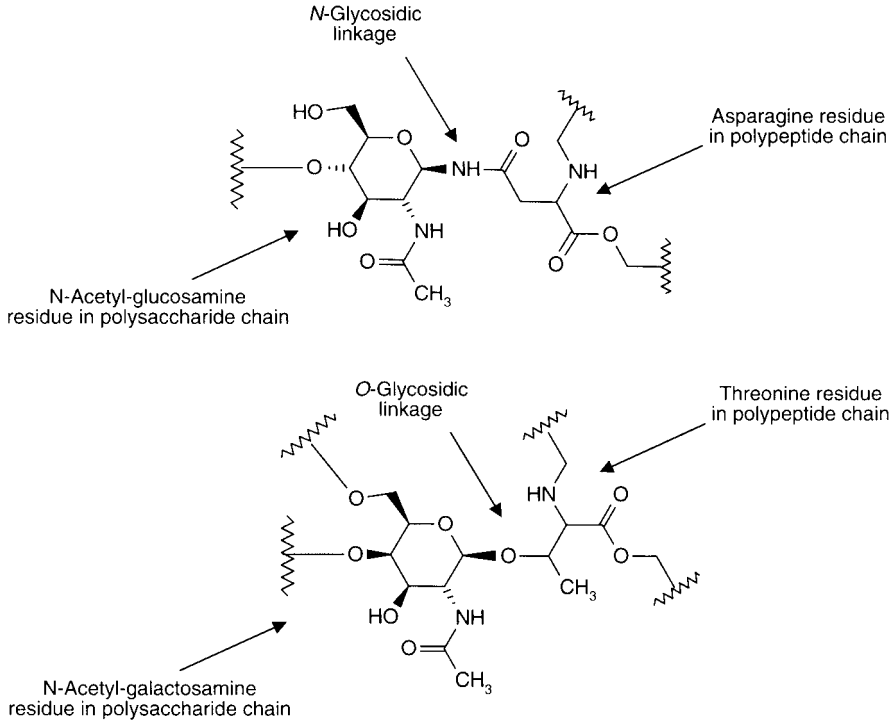


Figure 1.34 Common attachment points for polysaccharide chains on glycoproteins.

Carbohydrate also is an important constituent of many biological molecules. Polysaccharides may be found covalently conjugated to proteins and lipids, forming glycoproteins, proteoglycans, glycolipids, and lipopolysaccharides. Such glycoconjugates (glycans) are produced in the cell through controlled, enzymatic processes. With proteins, the modification occurs after translational synthesis of the polypeptide chain at the ribosome.

Proteins newly synthesized on ribosomes, may be transported to the Golgi apparatus where specific glycosyl transferases catalyze the coupling of monosaccharides to the polypeptide chains. Glycoproteins and mucoproteins are formed by the coupling of polysaccharides through *o*-glycosidic linkages to serine, threonine, or hydroxylysine in addition to *N*-glycosidic linkages with the amide side chain group of asparagine (Figure 1.34). For reviews of glycoconjugate structure and function, see Hynes (1987); Lennarz (1980); Jentoft (1990); Steer and Ashwell (1986), and the entire issue of Science Vol. 291, March 23, 2001 (special edition on Carbohydrates and Glycobiology).

The structure of most glycoprotein carbohydrate consists of a complex, branched heteropolysaccharide with the sugars mannose, *N*-acetyl glucosamine, sialic acid, galactose, and L-fucose being prevalent. Asparagine-linked polysaccharides are well characterized and are known to be constructed of a core unit consisting of three mannose residues and two *N*-acetyl glucosamine (GlcNAc) residues. The GlcNAc residues are bound to the Asp side chain amide nitrogen through a β1 linkage (Kornfield and Kornfield, 1985). The three mannose groups then usually form the first branch point in the oligosaccharide chain. Figures 1.35 and 1.36 show the

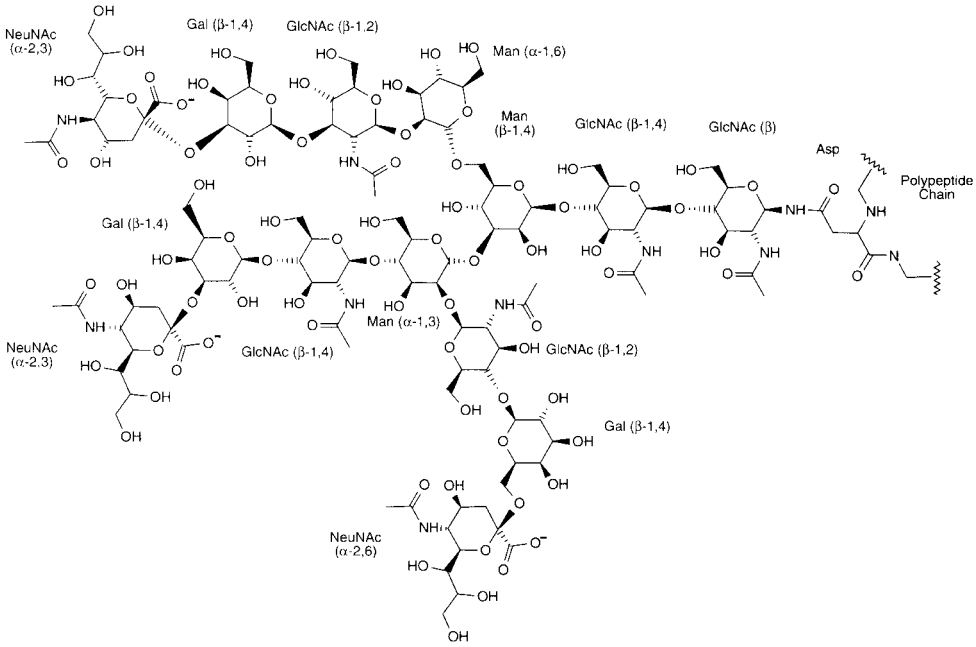


Figure 1.35 The complex structure of an asparagine-linked polysaccharide. Note the branched nature of the polymer with terminal sialic acid residues on each chain.

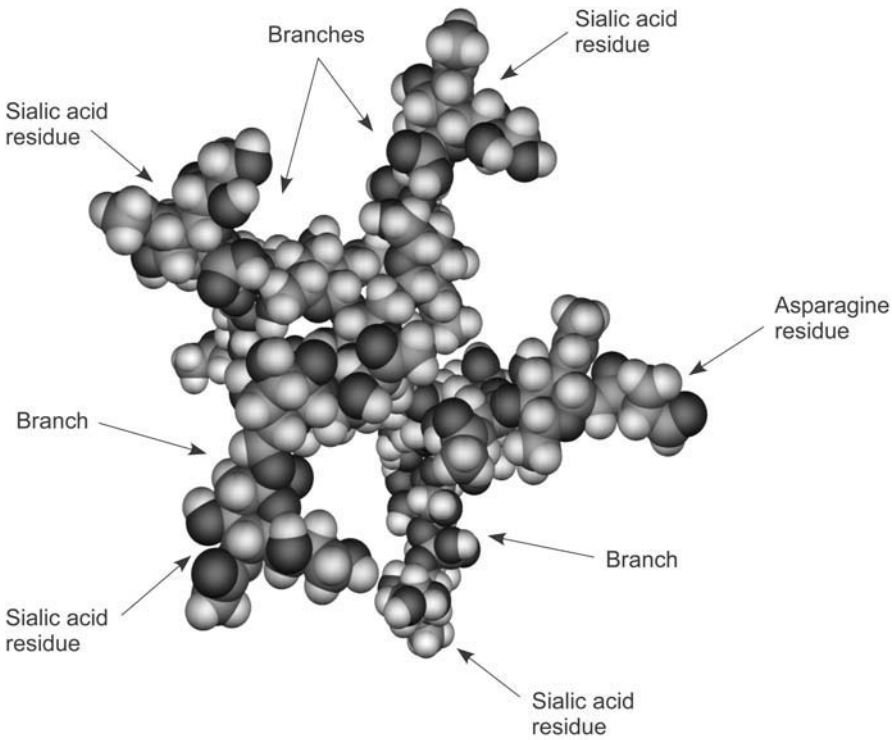


Figure 1.36 A space-filling model of an *N*-linked glycan showing four branch points.

chemical makeup of a typical *N*-linked glycan and a space-filling model of a glycan's molecular structure. Much of the detailed structural knowledge of glycoconjugates is developed using controlled chemical or enzymatic degradation of the polysaccharides followed by analysis by gas chromatography and mass spectrometry (Vliegenthart *et al.*, 1983; Sweeley and Nunez, 1985; McCleary and Matheson, 1986; Biermann and McGinnis, 1989).

The content by weight of carbohydrate in glycoproteins may vary from only a few percent to as much as 70 percent in some proteins in mucous secretions. Although the exact function of the polysaccharide in most glycoproteins is unknown, in some cases it may provide hydrophilicity, recognition, and points of noncovalent interaction with other proteins through lectin affinity binding. Glycosylation also contributes to the correct folding of proteins after translation, probably by assuring that certain amino acid regions end up at the surface of the protein structure. In addition, extensive polysaccharide modification is helpful in preventing proteolytic digestion of the underlying polypeptide chain.

Another form of post-translational modification that may add carbohydrate to a polypeptide is non-enzymatic glycation. This reaction occurs between the reducing ends of sugar molecules and the amino groups of proteins and peptides. The aldehyde group of a reducing sugar first forms a reversible Schiff's base linkage with the α-amino or ε-amino groups of the protein. This bond then can undergo an Amadori rearrangement to form a stable ketoamine derivative (Figure 1.37). The result is a blocked amine containing a sugar derivative with available

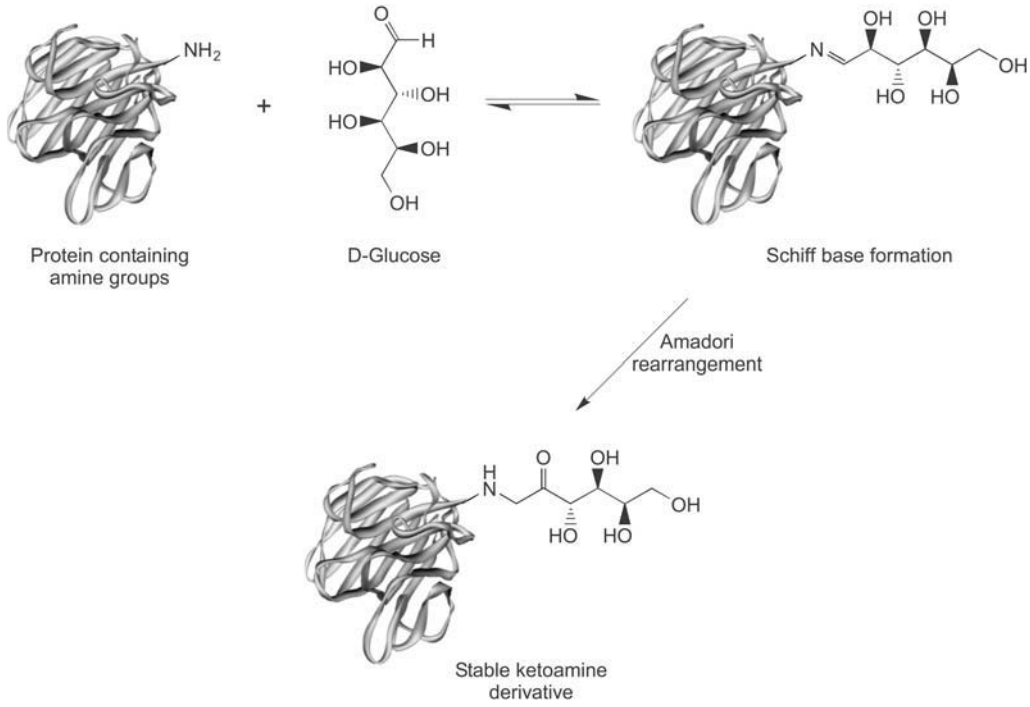


Figure 1.37 A reducing sugar may modify protein amine groups through Schiff base formation followed by an Amadori rearrangement to give a stable ketoamine product. Glucose is a common *in vivo* modifier of blood proteins through this process.

hydroxyl residues. This reaction commonly occurs with proteins continually exposed to reducing sugars, such as glucose in blood. The measurement of glycated hemoglobin is a clinically important parameter in the management of diabetes mellitus. Increases in the blood sugar level in diabetes cause concomitant increases in the level of non-enzymatic glycation of blood proteins. Measuring the relative amount of glycated hemoglobin provides the physician with information concerning a diabetic patient's blood glucose control.

2.2. Carbohydrate and Glycan Conjugation Methods

The presence of carbohydrate on biomolecules provides important points of attachment for modification and conjugation reactions. Coupling only through polysaccharide chains often can direct the reaction away from active centers or critical points in protein molecules, thus preserving activity. Crosslinking strategies involving polysaccharides or glycoconjugates usually involve a 2- or 3-step reaction sequence. If no reactive functionalities other than hydroxyl groups are present on the carbohydrate, then the first step is to create sufficiently reactive groups to couple with the functional groups of a second molecule.

Perhaps the easiest way specifically to target polysaccharides on glycoproteins is through mild sodium periodate oxidation. Periodate cleaves the carbon–carbon bond connecting adjacent hydroxyl groups in sugar residues to create highly reactive aldehyde functionalities (Section 4.4, this chapter). The level of oxidant addition can be adjusted to cleave selectively only certain sugars in the polysaccharide structure. A concentration of 1 mM sodium periodate at 0–4°C oxidizes sialic acid residues to aldehydes, leaving all other monosaccharides untouched. Increasing the concentration to 10 mM at room temperature, however, will cause oxidation of other sugars in the carbohydrate, including galactose and mannose residues in glycans. The generated aldehydes then can be used in coupling reactions with amine- or hydrazide-containing molecules to form covalent linkages. Amines react with formyl groups under reductive amination conditions using a suitable reducing agent such as sodium cyanoborohydride. The result of this reaction is a stable secondary amine linkage (Chapter 2, Section 5.3). Hydrazides spontaneously react with aldehydes to form hydrazone linkages, although the addition of a reducing agent greatly increases the efficiency of the reaction and the stability of the bond (Chapter 2, Section 5.1).

Oxidized glycoconjugates usually are stable enough to be stored in a freeze-dried state without loss of activity prior to a subsequent conjugation reaction, provided the protein itself is stable to lyophilization. Storage in solution, however, may cause slow polymerization if the molecule also contains amine groups, as in glycoproteins. Sometimes the protein can be treated to block its amines prior to periodate oxidation, as in the procedure often used with the enzyme horseradish peroxidase (HRP) (Chapter 26, Section 1.1), thus eliminating the potential for self-conjugation. Even in the absence of amines, periodate oxidized HRP may polymerize due to the Mannich reaction (Chapter 2, Section 5.4).

If the second molecule to be coupled to the oxidized-glycoconjugate already has the requisite amines or hydrazide groups, then directly mixing the two components together in the presence of a reductant is all that is needed to form the conjugate. This is an example of a 2-step procedure. However, if the second molecule possesses none of the appropriate functionalities for coupling, then modifying it to contain amine or hydrazide groups must be done prior to the conjugation reaction (see Sections 4.3 and 4.5, this chapter). Thus, a 3-step protocol results.

The use of other functionalities (either indigenous or created) on polysaccharide molecules to effect a crosslinking reaction can be done in similar 2- or 3-step strategies.

Occasionally, it is important to conjugate a polysaccharide-containing molecule to another molecule while retaining, as much as possible, the carbohydrate's original chemical and three-dimensional structure. For instance, in the preparation of immunogen conjugates by coupling a polysaccharide molecule to a carrier, care should be taken to preserve the structure of the carbohydrate to assure antibody recognition of the native molecule. In this case, periodate-oxidative techniques may not be the best choice to effect crosslinking due to the potential for extensive ring opening throughout the chain. Under controlled conditions, however, where periodate is carefully used in limiting quantities, this method has proved successful in creating oligosaccharide–carrier conjugates (Anderson *et al.*, 1989).

Retention of native carbohydrate structure also is important in applications that utilize the conjugated polysaccharide in binding studies with receptors or lectins. In these cases, the carbohydrate should be modified at limited sites, preferentially only at its reducing end. Section 4.6 of this chapter discusses glycan conjugation techniques in greater detail.

3. Modification of Nucleic Acids and Oligonucleotides

The nucleic acid polymers DNA and RNA form the most basic units of information storage within cells. The conversion of DNA's unique information code into RNA and proteins is the fundamental step in controlling all cellular processes. Targeting segments of this encoded data with labeled probes that are able to bind to specific genetic regions allows detection, localization, or quantification of discrete oligonucleotide sequences. This targeting capability is made possible by the predictable nature of nucleic acid interactions. Despite the complexity of the genetic code, the base-pairing process that causes one oligonucleotide to bind to its complementary sequence is rather simple to predict and decipher. Nucleic acids are the only type of complex biological molecule wherein their binding properties can be fully anticipated and incorporated into synthetic oligonucleotide probes. Thus, a short DNA segment can be synthetically designed and used to target and hybridize to a complementary DNA strand within much larger chromosomal material or extracted genomic DNA. If the small oligonucleotide is labeled with a detectable component that doesn't interfere in the base-pairing process, then the targeted DNA can be identified or assayed.

Bioconjugate techniques involving nucleic acids are becoming one of the most important application areas of crosslinking and modification chemistry. With the secrets of the genetic code now revealed by such mammoth efforts as the Human Genome Project, knowledge of the DNA sequence which governs specific protein expression is leading to diagnostic tests able to assess the presence of critical genetic markers associated with certain disease states. To test for particular target sequences, complementary oligonucleotide probes are used that possess conjugated enzymes, fluorophores, haptens, radiolabels, or other such groups which can be used to detect a hybridization signal. Such oligonucleotide conjugates can be used to discover target sequences in blots, electrophoresis gels, tissues, cells, immobilized to surfaces, or in solution.

The power and advantages of assessing cellular processes at their most fundamental level is propelling the science of oligonucleotide probe detection into one of the most prominent positions in bioconjugate chemistry. Oligonucleotide arrays containing hundreds or thousands of

tests now are done routinely to monitor different aspects of genetic information—all with the use of specific oligonucleotide probes.

In this section, the chemistry and structure of nucleic acids and oligonucleotides is discussed with a view to creating functional conjugates with detectable molecules. The corresponding strategies and protocols associated with DNA or RNA modification and conjugation can be found in Chapter 27.

3.1. Polynucleotide Structure and Functionality

Nucleic acid polymers are characterized by the types of base residues present and the structure of their sugar backbone. The bases are nitrogenous ring compounds consisting of either purine or pyrimidine derivatives. A purine is a fused-ring compound containing one 6-membered ring attached to a 5-membered ring, whereas a pyrimidine consists of a single 6-membered ring structure (Figure 1.38).

Nucleic acids can contain of any one of three kinds of pyrimidine ring systems (uracil, cytosine, or thymine) or two types of purine derivatives (adenine or guanine). Adenine, guanine, thymine, and cytosine are the four main base constituents found in DNA. In RNA molecules, three of these four bases are present, but with thymine replaced by uracil to make up the fourth. Some additional minor derivatives are found in messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), particularly the N^4,N^4-dimethyladenine and N^7-methylguanine varieties.

Nucleic acid sugar residues are attached to the associated base units in an *N*-glycosidic bond, involving the No. 1 nitrogen of pyrimidine bases or the No. 9 nitrogen of purines directly linked to the No. 1 carbon of the monosaccharide derivative (Figure 1.39). The sugar group consists of either a β-D-ribose unit (found in RNA) or a β-D-2-deoxyribose unit (in DNA) (Figure 1.40). In mRNA and rRNA, a minor sugar derivative, a 2′-*O*-methylribosyl group, also is found.

The nomenclature of nucleic acid chemistry further characterizes the structure of the associated groups. A *nucleoside* contains only a base group and an attached sugar. A *nucleotide* consists of a base and a sugar plus a phosphate group. At this point, the naming system gets somewhat confusing due to the fact that the nucleoside name is a derivative of the base name. Table 1.2 shows this relationship and their associated abbreviations (which are simpler to remember).

In each nucleotide monomer of DNA or RNA molecules, a phosphate group is attached to the C-5 hydroxyl of each sugar residue in an ester (anhydride) linkage. These phosphate groups in turn are linked in diester bonds to neighboring sugar groups of adjacent nucleotides

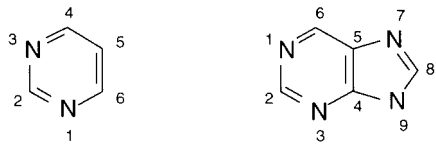


Figure 1.38 The pyrimidine and purine ring structures common to nucleic acids.

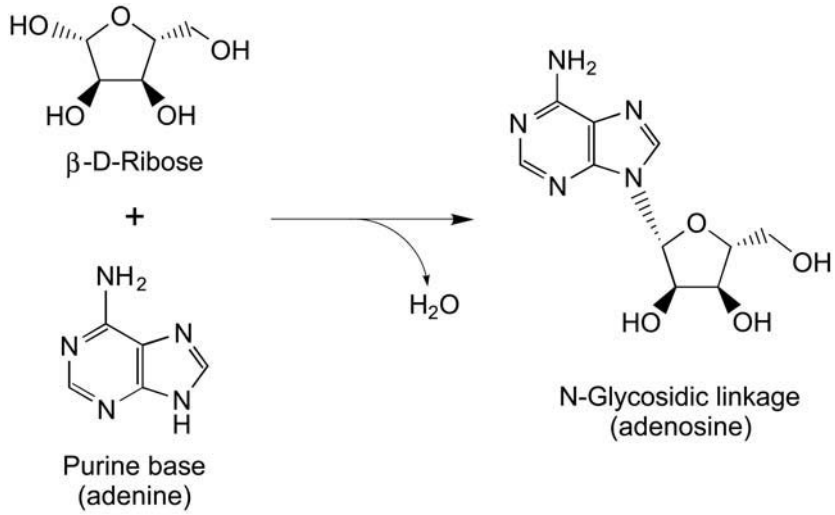


Figure 1.39 The formation of an *N*-glycosidic bond links the base unit of nucleic acids to the associated ribose derivative.

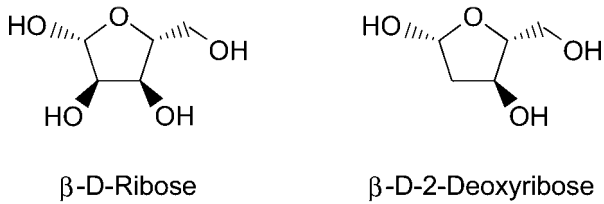


Figure 1.40 The two forms of sugar residues commonly found in nucleic acids. β-D-Ribose is the sugar constituent of RNA, while β-D-2-deoxyribose is a component of DNA.

Table 1.2 Nucleic Acid Nomenclature

Base name	Nucleoside name[a] (base + sugar)	Nucleotide name[b] (base + sugar + phosphate)
Adenine	Adenosine	Adenosine monophosphate (AMP)
Guanine	Guanosine	Guanosine monophosphate (GMP)
Cytosine	Cytidine	Cytidine monophosphate (CMP)
Thymine	Thymidine	Thymidine monophosphate (TMP)
Uracil	Uridine	Uridine monophosphate (UMP)

[a]For deoxyribose nucleosides, add "deoxy" before the nucleoside name. For example, adenosine becomes deoxyadenosine.
[b]For the presence of two phosphate groups, the names are changed to diphosphate. For three phosphate groups, the terminology is triphosphate.

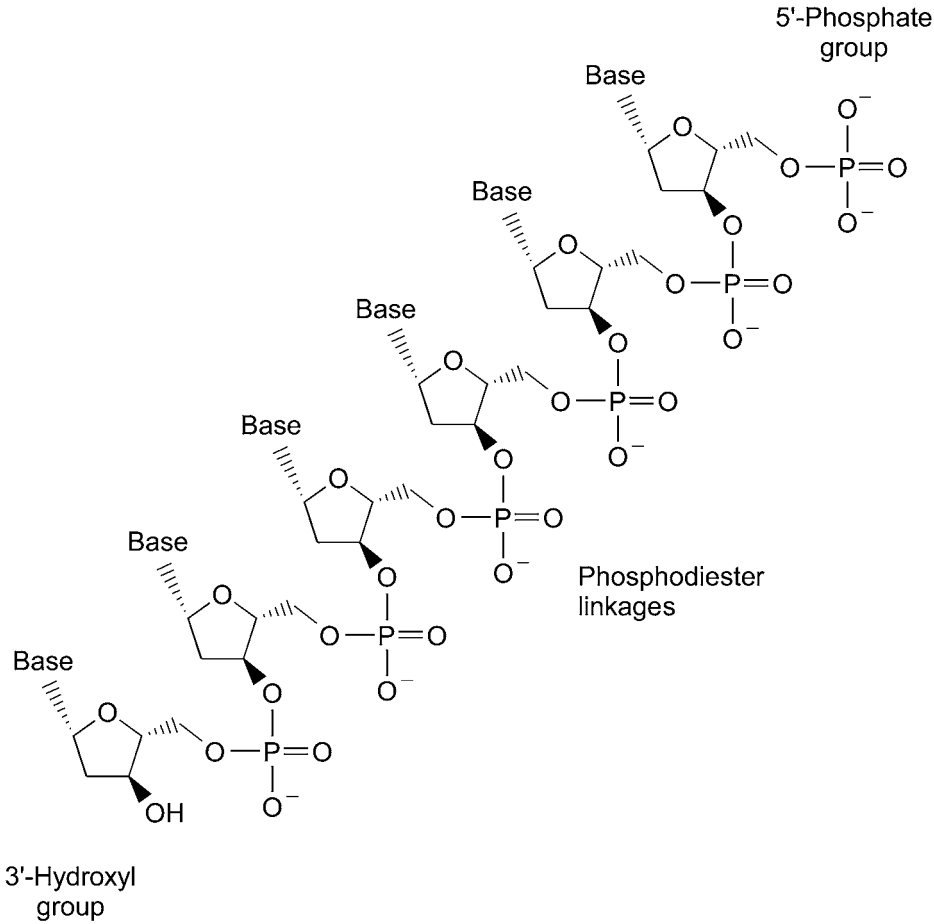


Figure 1.41 Polynucleotides are formed through phosphodiester bonds linking the associated sugar groups together. In DNA, the 3′-hydroxyl of one deoxyribose unit is bound to the 5′-hydroxyl of the next, creating direction in the polymer backbone.

through their 3′-ribosyl hydroxyl to create the oligonucleotide polymer backbone (Figure 1.41). Thus, the phosphate–sugar repeating unit produces the linear sequence within the DNA or RNA structure, while the four types of base units protrude out from this backbone, creating the unique code making up the genetic information.

Nucleotide Functional Groups

Chemical attachment of a detectable component to an oligonucleotide forms the basis for constructing a sensitive hybridization reagent. Unfortunately, the methods developed to crosslink or label other biological molecules such as proteins do not always apply to nucleic acids. The major reactive sites on proteins involve primary amines, sulfhydryls, carboxylates, or phenolates—groups that are relatively easy to derivatize. RNA and DNA contain none of these functionalities.

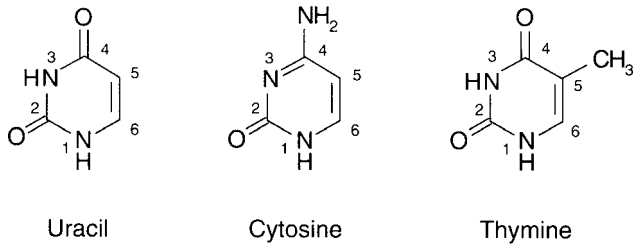


Figure 1.42 The three pyrimidine bases common to nucleic acid construction. Cytosine and thymine are found in DNA, while in RNA, uracil residues replace thymine. The associated sugar groups are bound in *N*-glycosidic linkages to the N-1 nitrogen.

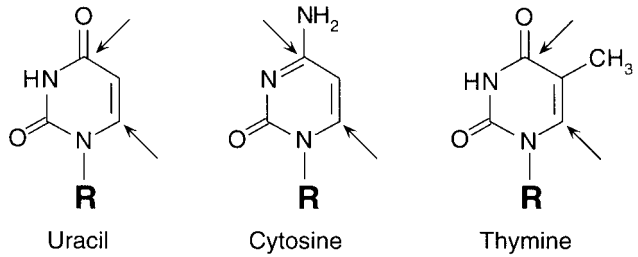


Figure 1.43 Pyrimidine bases are subject to nucleophilic displacement reactions primarily at the C-4 and C-6 positions.

They also are relatively unreactive directly with many of the common bioconjugate reagents discussed in Part II.

However, there are particular sites that can be modified on the bases, sugars, or phosphate groups of nucleic acids to produce derivatives able to couple with a second molecule. The chemistry is almost entirely unique to DNA and RNA work, but once mastered, the process of conjugation can be done with the same ease as with protein molecules.

The following sections discuss the major constituents of oligonucleotides with special emphasis on the chemical sites useful for bioconjugation.

Cytosine, Thymine, and Uracil Residues

The pyrimidine base units cytosine, thymine, and uracil contain 6-membered nitrogenous ring structures with various points of unsaturation. Thymine and uracil are similar, containing the same double bond between carbons 5 and 6 and the same two ketone groups on C-2 and C-4 of the ring, but differ only in the presence of a methyl group on the No. 5 carbon of thymine. Cytosine, by contrast, contains an additional site of unsaturation between carbons 3 and 4 as well as an amine group on C-4 instead of a ketone (Figure 1.42).

Figure 1.43 indicates major sites of reactivity within the ring structures for nucleophilic displacement reactions. Cytosine, thymine, and uracil all react toward nucleophilic attack at the same two sites, the C-4 and C-6 positions. The presence of powerful nucleophiles, even at neutral pH, can lead to significant base modification or cleavage with pyrimidine residues (Debye, 1947). For instance, hydrazine spontaneously adds to the 5,6-double bond, initiating further ring reactions,

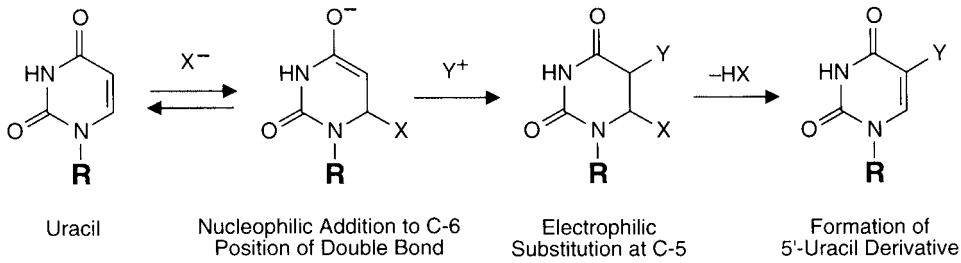


Figure 1.44 Nucleophilic addition at C-6 of the pyrimidine double bond can cause electrophilic substitution to occur at the C-5 position.

which eventually leads to oligonucleotide degradation. A similarly strong nucleophile, hydroxylamine, is almost entirely specific for modifying pyrimidines. It too can add to the 5,6-double bond, creating a 6-hydroxylamino derivative. In general, the pyrimidines can undergo reactions at the 5,6-double bond leading to a stable modification at the C-5 position (Figure 1.44).

Addition of a nucleophile to the C-6 position of cytosine often results in fascile displacement reactions occurring at the N-4 location. With hydroxylamine attack, nucleophilic displacement causes the formation of an N_4-hydroxy derivative. A particularly important reaction for bioconjugate chemistry, however, is that of nucleophilic bisulfite addition to the C-6 position. Sulfonation of cytosine can lead to two distinct reaction products. At acid pH wherein the N-3 nitrogen is protonated, bisulfite reaction results in the 6-sulfonate product followed by spontaneous hydrolysis. Raising the pH to alkaline conditions causes effective formation of uracil. If bisulfite addition is done in the presence of a nucleophile, such as a primary amine or hydrazide compound, then transamination at the N-4 position can take place instead of hydrolysis (Figure 1.45). This is an important mechanism for adding spacer arm functionalities and other small molecules to cytosine-containing oligonucleotides (see Chapter 27, Section 2.1).

Electrophilic reagents also can modify the pyrimidine rings of nucleic acids. Alkylation and acylation reactions can take place at several sites on all three bases. Figure 1.46 illustrates the principal locations where electrophilic attack can occur. In particular, the heteroatoms (oxygen and nitrogen) are the best positions of high electron density, therefore functioning as nucleophiles in reaction processes. Of the pyrimidine residues, however, it is the N-3 position of cytosine derivatives that is the most susceptible to alkylation. Reactions can occur with ethylenimine compounds (Section 4.3, this chapter), alkyl halogens (Chapter 2, Section 2.1), epoxides (Chapter 2, Section 1.7), and many other strong alkylating agents (for review, see Brown, 1974).

Acylation reactions can be done at the nucleophilic sites on pyrimidines using activated forms of carboxylic acids. Acylation of functional groups in nucleotides typically is used for protection during synthesis (Reese, 1973). However, for bioconjugate applications, the reactivity of native groups on pyrimidines is not as great as that obtained using an amine-terminal spacer derivative, such as those described in Chapter 27, Section 2.1. Yields and reaction rates are typically low for direct acylation or alkylation of pyrimidine bases, especially in aqueous environments.

The N-3 position of uracil also can be modified with carbodiimide reagents. In particular, the water-soluble carbodiimide CMC [1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide, as the metho *p*-toluene sulfonate salt] can react with the N-3 nitrogen at pH 8 to give an unstable, charged adduct. The derivative is reversible at pH 10.5, regenerating the original nucleic acid base (Figure 1.47). Cytosine is unreactive in this process.

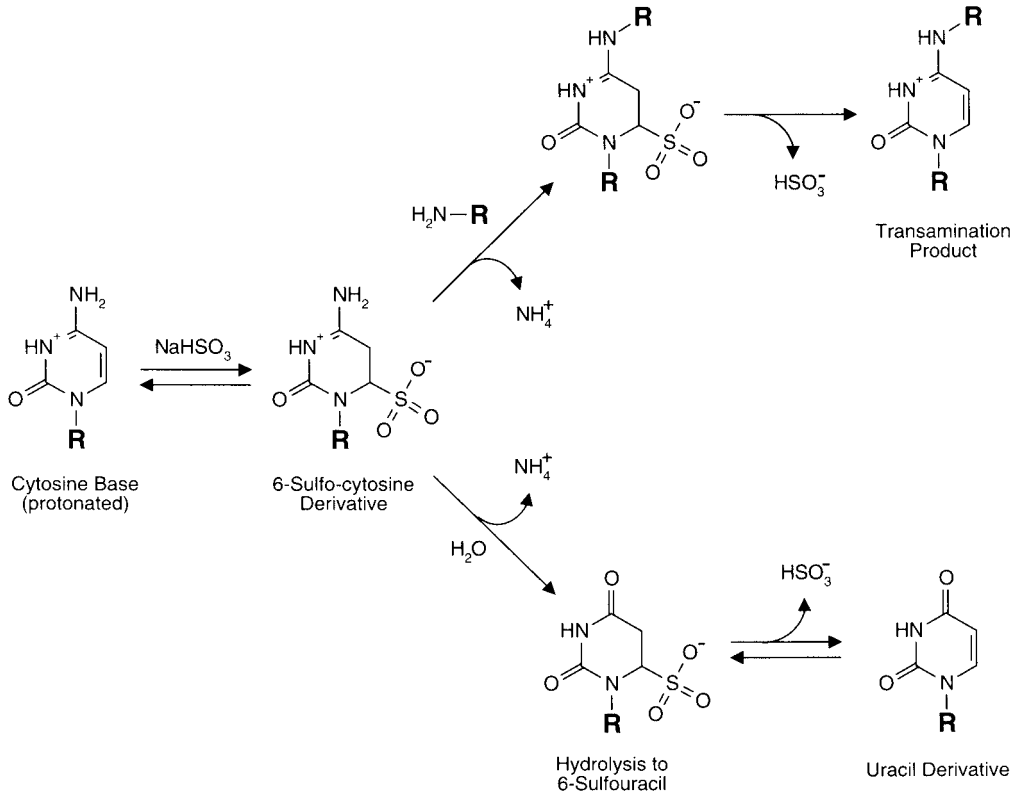


Figure 1.45 Reaction of bisulfite with cytosine bases is an important route of derivatization. It can lead to uracil formation or, in the presence of an amine (or hydrazide) containing compound, transamination can occur, resulting in covalent modification.

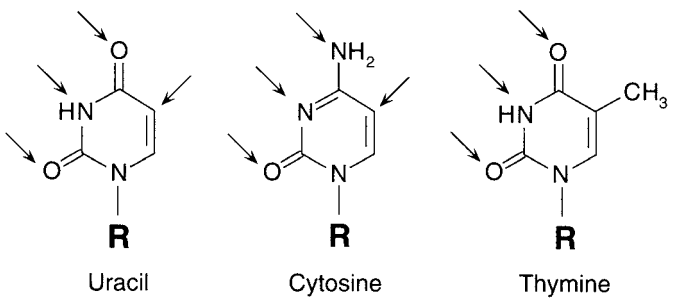


Figure 1.46 Potential sites of electrophilic attack on pyrimidine bases.

Halogenation of pyrimidine bases may be done with bromine or iodine. Bromination occurs at the C-5 of cytosine, yielding a reactive derivative, which can be used to couple diamine spacer molecules by nucleophilic substitution (Figure 1.48) (Traincard *et al.*, 1983; Sakamoto *et al.*, 1987; Keller *et al.*, 1988). Other pyrimidine derivatives also are reactive to bromine compounds

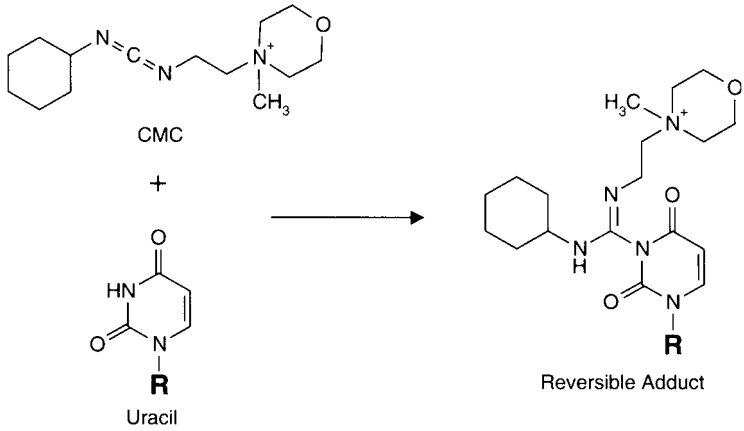


Figure 1.47 The carbodiimide CMC can react with the N-3 nitrogen to yield a reversible product.

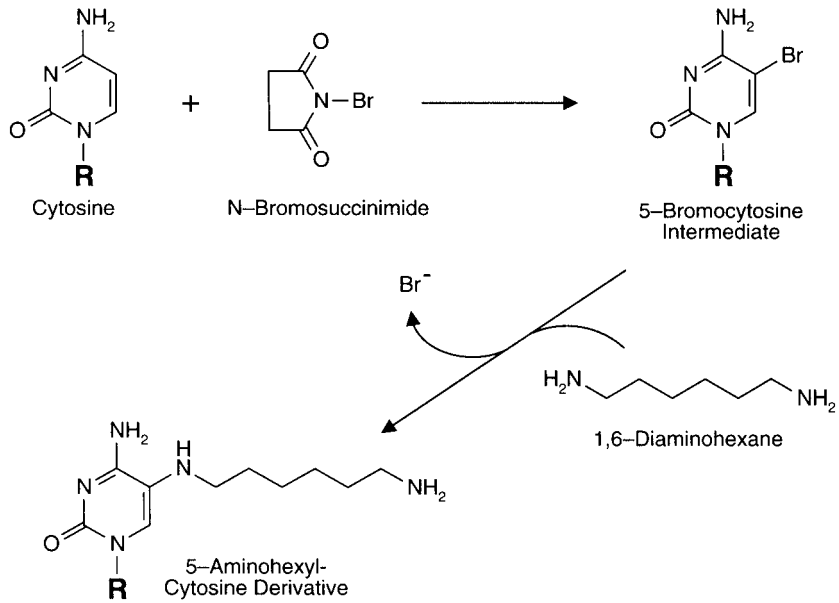


Figure 1.48 Cytosine bases are susceptible to bromination at the C-5 double bond position, resulting in active intermediates capable of reacting with amine nucleophiles.

at the C-5 position. Either an aqueous solution of bromine or the compound *N*-bromosuccinimide can be used for this reaction. The brominated derivatives then can be used to couple amine-containing compounds to the pyrimidine ring structure (Chapter 28, Section 2.1).

Other reactions characterized for pyrimidine residues include mercuration at C-5 of cytosine or uracil (Hopman *et al.*, 1986), cycloaddition to the 5,6-double bond of thymine and uracil (Cimino *et al.*, 1985), and thiolation at the C-4 amino group of cytosine (Malcom and Nicolas, 1984).

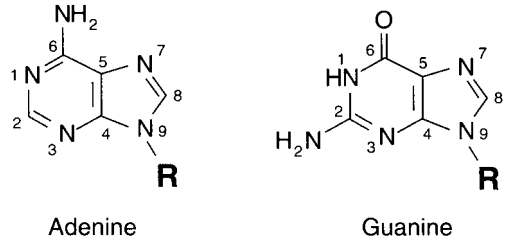


Figure 1.49 The structures of the common purine bases of RNA and DNA. The associated sugar groups are bound in *N*-glycosidic linkages to the N-9 position.

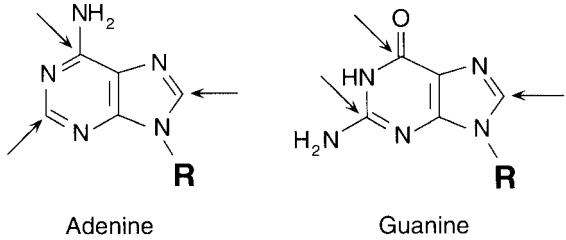


Figure 1.50 Primary nucleophilic displacement sites on purine bases.

Adenine and Guanine Residues

The purine bases of nucleic acids are constructed of a two-ring system made from a pyrimidine-type, 6-membered ring fused with a 5-membered imidazole ring. Adenine and guanine are present in both RNA and DNA. They differ in their 6-membered ring structures by an additional point of unsaturation between C-6 and N-1 (in adenine) and by the presence of amine or ketone groups attached to C-2 or C-6 (Figure 1.49). Attachment to ribose or deoxyribose in nucleosides is made through an *N*-glycosidic linkage at N-9 of the imidazole ring on either purine.

As in the case of pyrimidine bases discussed previously, adenine and guanine are subject to nucleophilic displacement reactions at particular sites on their ring structures (Figure 1.50). Both compounds are reactive with nucleophiles at C-2, C-6, and C-8, with C-8 being the most common target for modification. However, the purines are much less reactive to nucleophiles than the pyrimidines. Hydrazine, hydroxylamine, and bisulfite—all important reactive species with cytosine, thymine, and uracil—are almost unreactive with guanine and adenine.

With purines, reaction with electrophilic species is the most important route to derivatization. Figure 1.51 identifies the major sites of electrophilic attack on adenine and guanine. On both bases it is the heteroatoms which make up the majority of sites. Alkylation reactions thus can occur at N-1, N-3, and N-7 in adenine or N-3 and N-7 in guanine. However, the greatest location of electron density (nucleophilicity) occurs at N-7 on the imidazole ring of guanine, followed by N-1 of adenine. According to Brown (1974), the order of reactivity of nucleosides toward alkylation by esters of strong acids is guanine > adenosine > cytidine >> uridine (nearly unreactive).

As with pyrimidines, the water-soluble carbodiimide CMC may react with guanine derivatives to give a reversible adduct at N-1 (Figure 1.52). Raising the pH to highly alkaline conditions regenerates the purine group. Adenine residues, however, display no reactivity in this process.

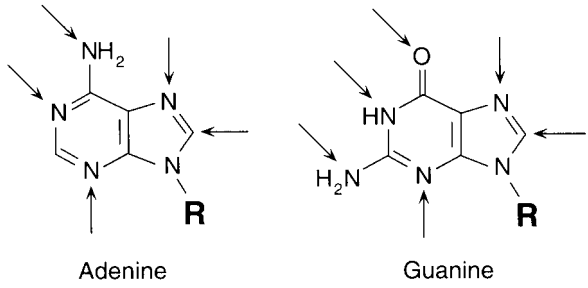


Figure 1.51 Electrophilic attack can occur at a number of sites on both purine bases.

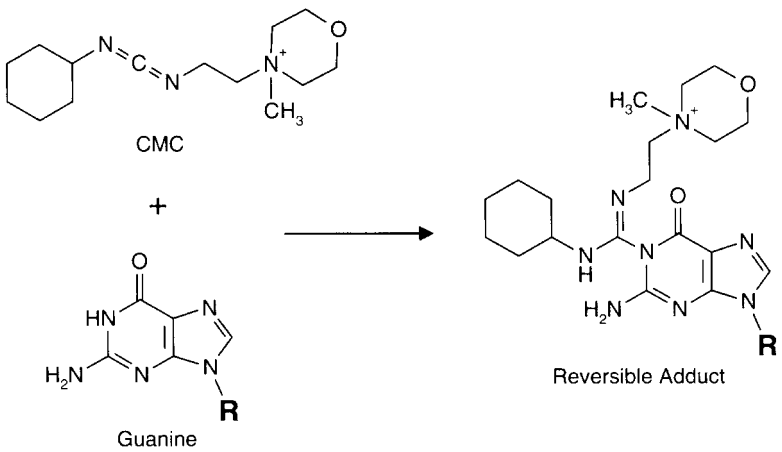


Figure 1.52 The carbodiimide CMC can react with guanine at the N-1 position to form a reversible complex.

One of the most important reactions of purines is the bromination of guanine or adenine at the C-8 position. It is this site that is the most common point of modification for bioconjugate techniques using purine bases (Figure 1.53). Either an aqueous solution of bromine or the compound *N*-bromosuccinimide can be used for this reaction. The brominated derivatives then can be used to couple amine-containing compounds to the pyrimidine ring structure by nucleophilic substitution (Chapter 27, Section 2.1).

Adenine also may undergo an additional reaction at its C-6 amine group using a Fischer–Dimroth rearrangement mechanism. Alkylation at N-1 can result in a rearrangement to give the C-6 alkylated product. The reaction at N-1 usually requires extended time to obtain good yields. For instance, alkylation with iodoacetic acid takes 5–10 days at pH 6.5. Under alkaline conditions and elevated temperatures, the 6-membered ring then is broken and reformed, resulting the 6-aminoalkylated product containing a terminal carboxylate group (Figure 1.54). The resultant acid can be used in further derivatization reactions to facilitate conjugate formation (Lowe, 1979).

An additional reaction reported for adenine involves the coupling of glutaraldehyde to the 6-amino group (Matthews and Kricka, 1988). However, reaction at this group with electrophilic reagents such as those discussed in Section 2 proceeds more slowly than that possible

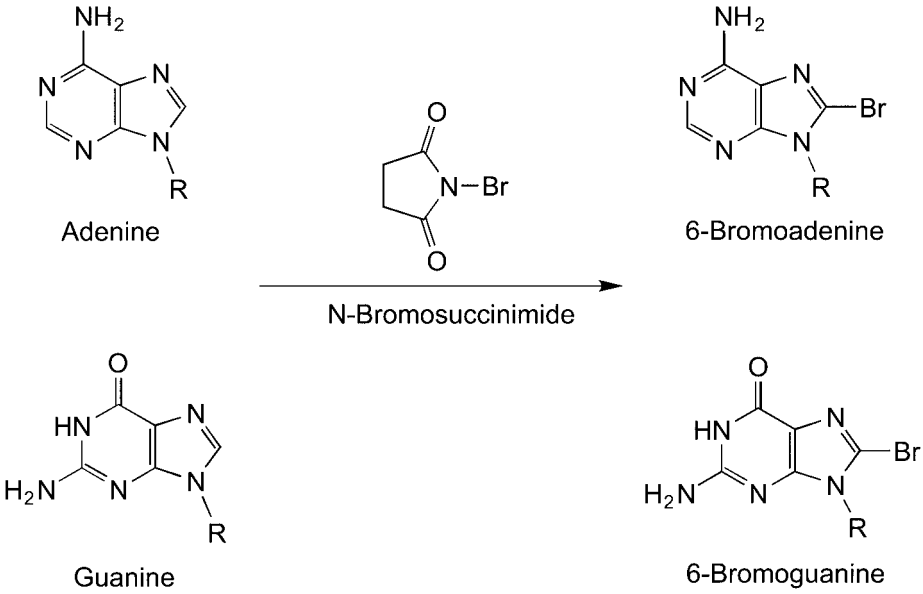


Figure 1.53 The purine bases are subject to bromination reactions at the C-8 position, forming an important reactive intermediate for derivatization purposes.

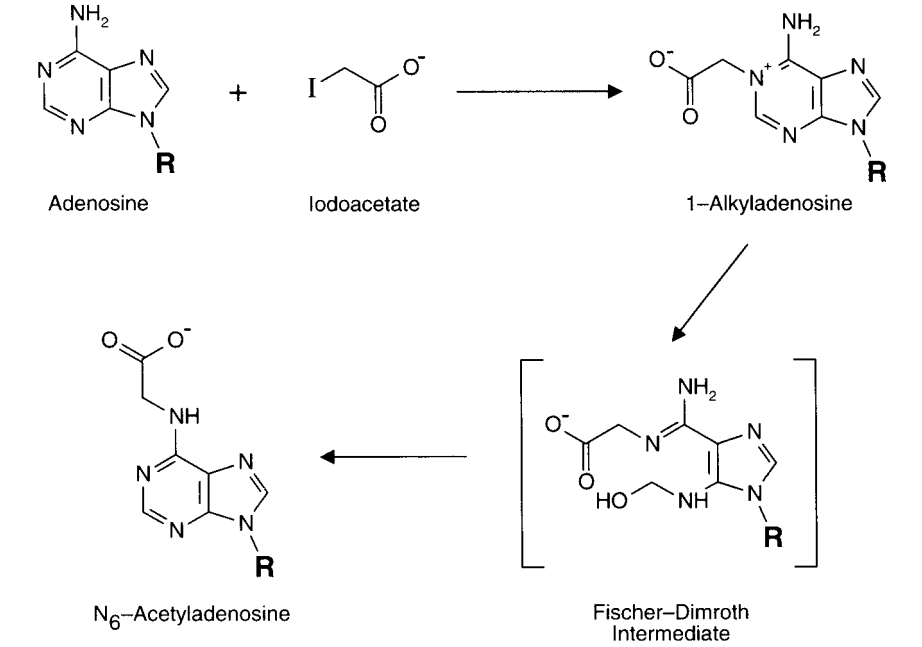


Figure 1.54 Alkylation reactions can occur at the N-1 position of adenosine, resulting in a Fischer–Dimroth rearrangement to yield an N_6 derivative.

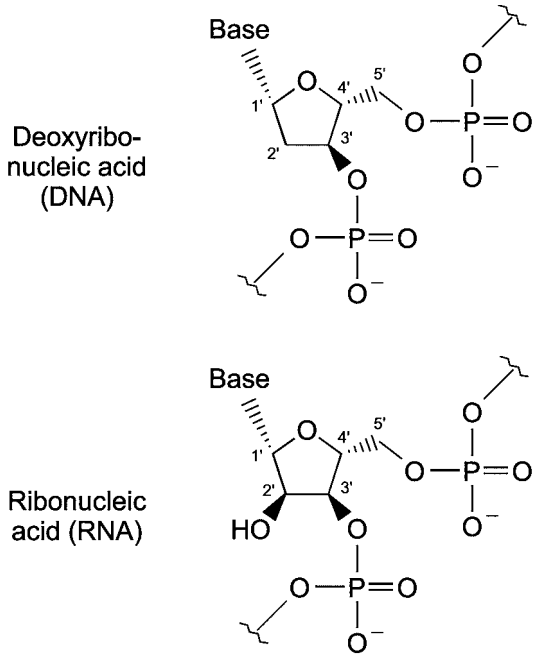


Figure 1.55 The similar structures of DNA and RNA basic units.

using a primary aliphatic amine. In general, bioconjugate chemistry done with nucleic acid bases involves the formation of an intermediate derivative containing a spacer arm terminating in an amine, sulfhydryl, or carboxylate to obtain acceptable reactivity and yields.

Sugar Groups

The sugar portion of oligonucleotides is a 5-carbon pentose occurring in one of two forms. In RNA, it is β-D-ribose in a ring structure. In DNA, the monosaccharide is β-D-2-deoxyribose, wherein the No. 2′-carbon of the ring lacks a hydroxyl group. An individual nucleotide will have its 1′-hydroxyl group of the ribose unit tied up in an *N*-glycosidic bond with the associated base and its C-5 hydroxyl group bound to phosphate in an ester linkage. If the nucleotide is of the deoxy form, then the only remaining hydroxyl is on the 3′-carbon of the sugar unit. Ribonucleic acids, by contrast, contain a diol group formed from the two hydroxyls on the 2′- and 3′-carbons of ribose (Figure 1.55). Polymers of nucleic acids are created through diester phosphate bonds, mainly connected between the 5′-hydroxyl of one sugar group and the 3′-hydroxyl of the next adjacent sugar. Thus, DNA contains no hydroxyl groups except the single one at the 3′-terminal of each strand. RNA has one hydroxyl at each nucleotide sugar unit and a diol group at the 3′-end.

Conjugation or modification reactions may be done through the 3′-hydroxyl group of deoxyribonucleic acids or the 2′,3′-diol of ribonucleic acids. Hydroxyls may be targeted for coupling using strong alkylating agents under alkaline conditions. Epoxide compounds (Chapter 2, Section 4.1) are particularly effective at modifying hydroxyl groups. The most common

method of conjugation through nucleotide sugar units, however, is periodate oxidation of the adjacent hydroxyls of ribonucleic acids. Treatment with periodate breaks the carbon–carbon bond between the two hydroxyl residues and creates two aldehyde groups (Seela and Waldeck, 1975). A procedure for oxidizing carbohydrates with sodium periodate can be found in Section 4.4, this chapter. This method can be used to create RNA conjugates through directed coupling only at the 3′-end or to immobilize ribonucleic acids such as ATP to insoluble supports for affinity chromatography (Lowe, 1979).

Phosphate Groups

The phosphate groups of nucleotides are joined to the 5′-hydroxyl group of the sugar component in an ester or anhydride linkage. Several forms of nucleoside phosphate compounds are possible, containing up to three esterified phosphate groups polymerized off the ribose or deoxyribose unit. The presence of these groups contributes an overall negative charge to the nucleotide—minus two for the terminal phosphate group and minus one for each internal phosphate under alkaline conditions. Multiple esterified phosphates contain considerable potential energy from their easily hydrolyzed anhydride bonds. This energy is the basis for many biochemical transformations in biological systems. It is the triphosphate form of nucleosides that is utilized in DNA and RNA synthesis *in vivo*. However, nucleoside triphosphates and diphosphates such as ATP and ADP have numerous contributions to cellular metabolism beyond just oligonucleotide construction. Controlled hydrolysis of their multiple phosphate ester bonds releases energy for many biological operations. Other derivatives of nucleoside phosphate compounds provide cofactors for enzymes (such as coenzyme A) or are involved in signal transduction processes (such as cyclic AMP [cAMP]). Figure 1.56 shows some of these common nucleoside phosphate derivatives.

The phosphate groups of nucleotides may be targeted for modification reactions using condensation agents such as carbodiimides. In aqueous environments, EDC (Chapter 3, Section 1.1) may be used to couple amine-containing compounds to the terminal phosphate group of an oligonucleotide, forming a phosphoramidate linkage. In DNA or RNA chains, the internal phosphate groups do not react under the pH conditions of the modification. In this way, the 5′-phosphate group may be specifically targeted for modification or conjugation, thus avoiding potential interference with hydrogen bonding interactions with complementary polynucleotide strands. Chapter 27, Sections 2.1 and 2.2 describe the use of this reaction in bioconjugate applications.

Another phosphate modification procedure that is effective at adding detectable components to oligonucleotide probes is to use a phosphoramidite derivative. The common method of automated oligonucleotide synthesis is to use phosphoramidite chemistry to add nucleotides to the growing sequence. A functionalized phosphoramidite nucleotide derivative can be added at particular points in the synthetic process to create labeled probes of known structure. Non-nucleotide phosphoramidites also may be used to produce modified probes containing fluorescent molecules, biotin, chelating groups, or spacer groups with amines for further derivatization. Most of these techniques require an automated DNA synthesizer. The methods of DNA modification during synthesis have been reviewed and are beyond the scope of this book (Beaucage and Iyer, 1993).

RNA and DNA Structure

The nucleotides forming RNA or DNA molecules are linked together in phosphodiester bonds with sugar–phosphate repeating units. The esters are directionally linked between the 3′-hydroxyl

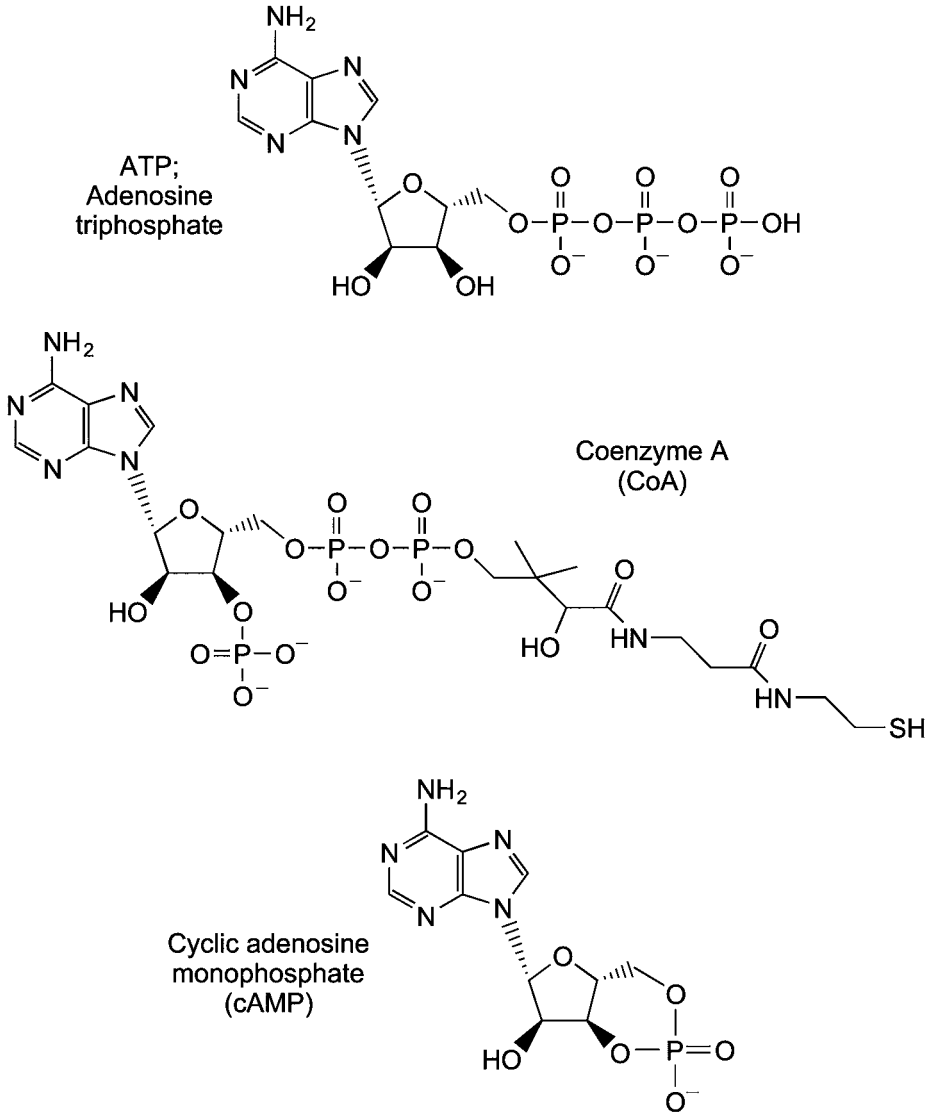


Figure 1.56 Nucleotide derivatives have additional functions *in vivo* beyond their role in oligonucleotide construction.

of one ribosyl group and the 5′-hydroxyl of the next. The fundamental step in cellular DNA synthesis involves the reaction of a deoxynucleoside triphosphate group with the 3′-end of an existing chain. The nucleotide sequence of a new strand is enzymatically controlled by use of a complementary chain as a template. Each new nucleotide addition is facilitated by the energy released through hydrolysis of two phosphates from the triphosphate group of the incoming nucleoside. The resulting succession of nucleotides encodes the message for protein synthesis, with each three-base code signaling a particular amino acid in a polypeptide sequence.

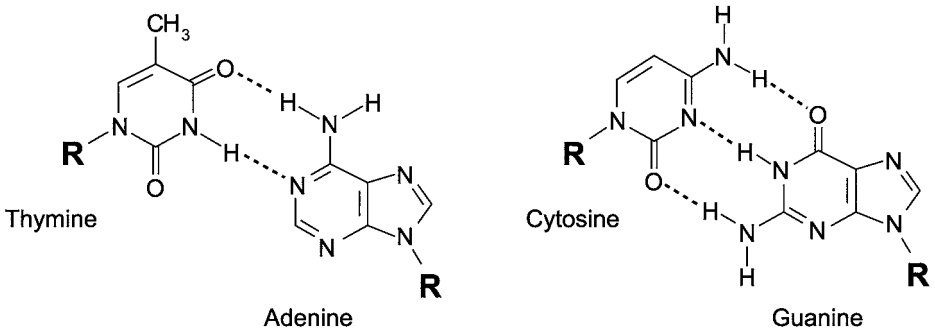


Figure 1.57 Base-pairing can occur between complementary bases in opposing oligonucleotide strands. These predictable interactions form the basis for using synthetic oligonucleotide probes to target particular DNA sequences.

Nucleotide bases projecting from the sugar–phosphate backbone of a polynucleotide are able to interact with other strands through hydrogen bonding. Hydrogen bonding can occur between cytosine and guanine base units in different strands of DNA through interaction of the C-2 ketone oxygen, the N-3 nitrogen, and C-4 amine groups of cytosine with the C-2 amine, N-1 nitrogen, and the C-6 ketone oxygen of guanine. In a similar fashion, thymine (or uracil) residues can hydrogen bond with adenine groups through the N-3 nitrogen and C-4 ketone oxygen of thymine interacting with the N-1 nitrogen and C-6 amine of adenine (Figure 1.57).

This specific base pairing capability of oligonucleotides defines the structure of complementary DNA molecules. In the classic Watson–Crick model, two complementary DNA strands interact in an antiparallel fashion to form a right-handed double helix. Thus, one chain runs in the 3′-to-5′ direction while the complementary chain runs in the 5′-to-3′ direction through the helical structure. This standard double helix, now called the B form, occurs often in aqueous solution and is the most stable structure under physiological conditions (Figure 1.58). However, there are several other forms that double-stranded DNA can take in solution. Another right-handed helical construction, the A form, can occur under nonaqueous conditions and is more compact than the B form. A completely different DNA structure, the Z form, is a left-handed helix that can occur in some segments containing an abundance of alternating pyrimidines and purines. Short segments of Z structure have been found in some cells. Finally, some rare DNA sequences can form triple-helical regions through normal and non-Watson–Crick base pairing.

Unlike the double-stranded nature of DNA, RNA molecules usually occur as single strands. This does not mean they are unable to base-pair as DNA can. Complementary regions within an RNA molecule often undergo base-pairing and form complex tertiary structures, even approaching the three-dimensional nature of proteins. Some RNA molecules, such as transfer RNA (tRNA) possess several helical areas and loops as the strand interacts with itself in complementary sections. Other hybrid molecules such as the enzyme RNase P contain protein and RNA portions. The RNA part is highly complex with many circles, loops, and helical regions creating a convoluted structure.

The predictable nature of DNA and RNA base pairing make their interactions the most defined of any biological system. The specific affinity of one strand for its complementary

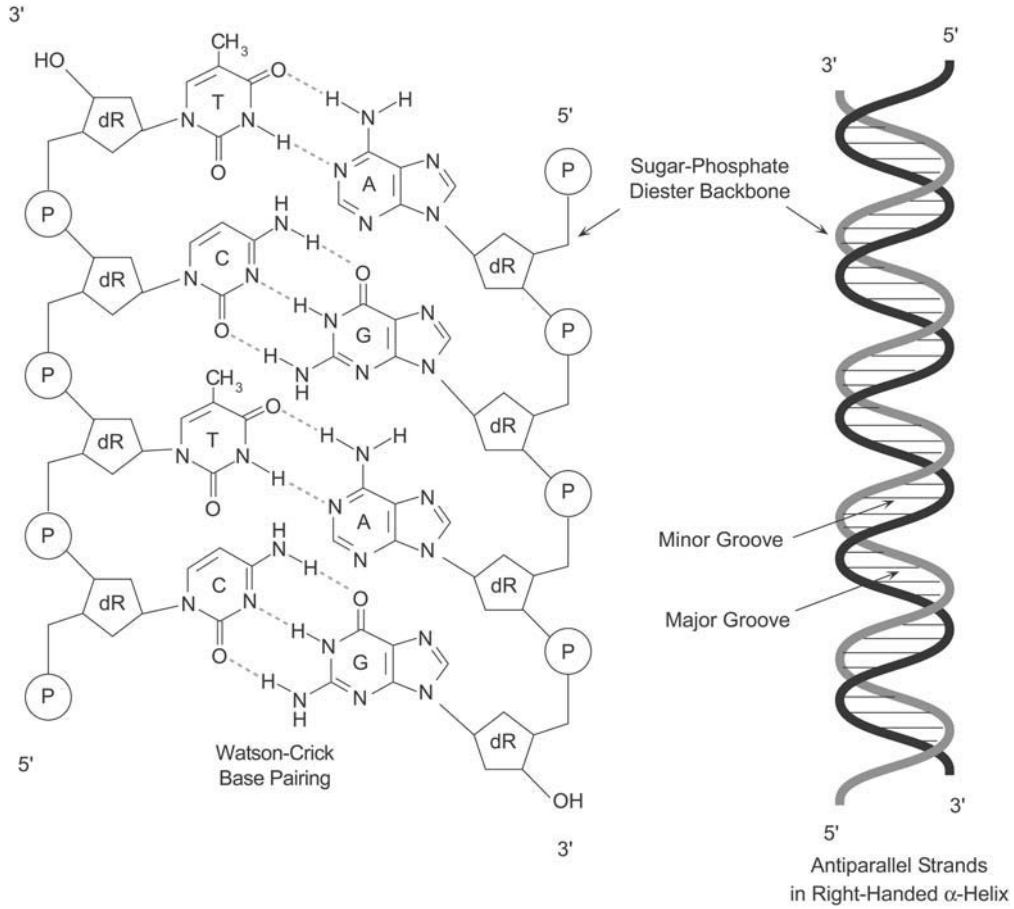


Figure 1.58 The classic Watson–Crick DNA double helix is formed through base-pairing interactions between two antiparallel strands. In physiological conditions, the two strands take on an α-helical shape with about 10 bp per turn of the helix. The phosphate–sugar backbone of the helix faces outward, while hydrogen bonding between opposing bases occurs in the middle of the wrapped strands. This configuration creates minor and major grooves between the phosphate–sugar backbones, potentially exposing the internal bases to interactions with other molecules.

sequence makes it possible to target genetic markers with extreme accuracy. Synthetic segments of RNA or DNA can be used to detect or quantify their complementary targets, even in highly dilute environments containing many other oligonucleotide molecules. If the oligonucleotide probe is labeled with a highly detectable component, then specific base pairing interactions can be assayed. This ability has created an extensive utilization of labeled probes in molecular biology. Detection of target DNA or RNA can be done in cells, tissue sections, blots, electrophoresis gels, after amplification by polymerase chain reaction (PCR) techniques, or in solution. The ability to detect single-copy genes through the use of labeled oligonucleotide probes will make this field one of the leading application areas for bioconjugate techniques.

3.2. Polynucleotide Crosslinking Methods

The unique properties of oligonucleotides create crosslinking options that are far different from any other biological molecule. Nucleic acids are the only major class of macromolecule that can be specifically duplicated *in vitro* by enzymatic means. The addition of modified nucleoside triphosphates to an existing DNA strand by the action of polymerases or transferases allows addition of spacer arms or detection components at random or discrete sites along the chain. Alternatively, chemical methods that modify nucleotides at selected functional groups can be used to produce spacer arm derivatives or activated intermediates for subsequent coupling to other molecules.

Thus, both chemical and enzymatic derivatization techniques can be used to form oligonucleotide probes of high activity in hybridization assays. The main consideration for successful polynucleotide crosslinking, as in other bioconjugate applications, is to avoid probe inactivation during the modification or conjugation process. Since the purpose in constructing a DNA or RNA probe is to hybridize to a complementary oligonucleotide through hydrogen bond interactions, any derivatization procedure which significantly interferes with Watson–Crick base pairing should be avoided. This means that a large amount of base derivatization along a polynucleotide chain has potential for causing obstructions in the hybridization process, sometimes dramatically reducing or eliminating base pairing efficiency. In general, base modifications within an oligonucleotide probe should be limited to no more than about 30–40 sites per 1,000 bases to maintain hybridization ability.

By contrast, derivatization at the ends of an oligo or at the sugar–phosphate backbone usually produces little interference in base pairing. Conjugates may be created by enzymatic polymerization of functionalized nucleoside triphosphates off the 3′-end or by chemical modification of the 5′-phosphate group with minimal to no interference in hybridization potential. The application of these strategies to creating labeled oligonucleotide probes is discussed in Chapter 27.

4. Creating Specific Functionalities

It is often desirable to alter the native structure of a macromolecule to provide functional targets for modification or conjugation. The use of most reagent systems requires the presence of particular chemical groups to effect coupling. For instance, heterobifunctional crosslinkers contain two different reactive species that are directed against different functionalities. One target molecule has to contain chemical groups able to react with one end of the crosslinker, while the other target molecule must contain groups able to react with the other end. Occasionally, the required chemical groups are not present on one of the target molecules and must be created. This usually can be done by reacting an existing chemical group with a modification reagent that contains or produces the desired functionality upon coupling. Thus, an amine can be "changed" into a sulfhydryl or a carboxylate can be altered to yield an amine simply by using the appropriate reagent.

This same type of modification strategy also can be used to create highly reactive groups from functionalities of rather low reactivity. For instance, carbohydrate chains on glycoproteins can be modified with sodium periodate to transform their rather unreactive hydroxyl groups into highly reactive aldehydes. Similarly, cystine or disulfide residues in proteins can be selectively reduced to form active sulfhydryls, or 5′-phosphate groups of DNA can be transformed to yield modifiable amines.

Alternatively, spacer arms can be introduced into a macromolecule to extend a reactive group away from its surface. The extra length of a spacer can provide less steric hindrance to conjugation and often yields more active complexes.

The use of modification reagents to create specific functionalities is an important technique to master. In one sense, the process is like using building blocks to construct on a target molecule any desired functional groups necessary for reactivity. The success of many conjugation schemes depends on the presence of the correct chemical groups. Care should be taken in choosing a modification strategy, however, since some chemical changes will radically affect the native structure and activity of a macromolecule. A protein may lose its capacity to bind a specific ligand. An enzyme may lose the ability to act upon its substrate. A DNA probe may no longer be able to hybridize to its complementary target. In many cases, the potential for inactivation relates to changing conformational structures, blocking active sites, or modifying critical functional groups. Trial and error and careful literature searches are often necessary to optimize any modification tactic.

4.1. Introduction of Sulfhydryl Residues (Thiolation)

The sulfhydryl group is a popular target in many modification strategies. Crosslinking agents that have more than one reactive group often employ a sulfhydryl-reactive functionality at one end to direct the conjugation reaction to a particular part of a target macromolecule. The frequency of sulfhydryl occurrence in proteins or other molecules is usually low (or nonexistent) compared to other groups like amines or carboxylates. The use of sulfhydryl-reactive chemistries thus can restrict modification to only a limited number of sites within a target molecule. Limiting modification greatly increases the chances of retaining activity after conjugation, especially in sensitive proteins like some enzymes. Unfortunately, sulfhydryl groups often need to be generated (from reduction of indigenous disulfides) or created (from use of the appropriate thiolation reagent systems). The following sections describe the most popular techniques of creating these functionalities. Some of these reagent systems are specifically designed to form —SH groups, while others are crosslinkers that also can serve the dual purpose of sulfhydryl-generating agents.

Sulfhydryl groups are susceptible to oxidation and formation of disulfide crosslinks. To prevent disulfide bond formation, remove oxygen from all buffers by degassing under vacuum and bubbling an inert gas (i.e., nitrogen) through the solution. In addition, EDTA (0.01–0.1 M) may be added to buffers to chelate metal ions, preventing metal-catalyzed oxidation of sulfhydryls. Some proteins of serum origin (particularly bovine serum albumin (BSA)) contain so much contaminating metal ions (presumably iron from hemolyzed blood) that 0.1 M EDTA is required to prevent this type of oxidation.

Modification of Amines with 2-Iminothiolane (Traut's Reagent)

Perham and Thomas (1971) originally prepared an imidoester compound containing a thiol group, methyl 3-mercaptopropionimidate hydrochloride. The imidoester group can react with amines to form a stable, charged linkage (Chapter 2, Section 1.10), while leaving a sulfhydryl group available for further coupling (Figure 1.59). Traut *et al.* (1973) subsequently synthesized an analogous reagent containing one additional carbon, methyl 4-mercaptobutyrimidate. Later, this compound was found to cyclize as a result of the sulfhydryl group reacting with the intrachain

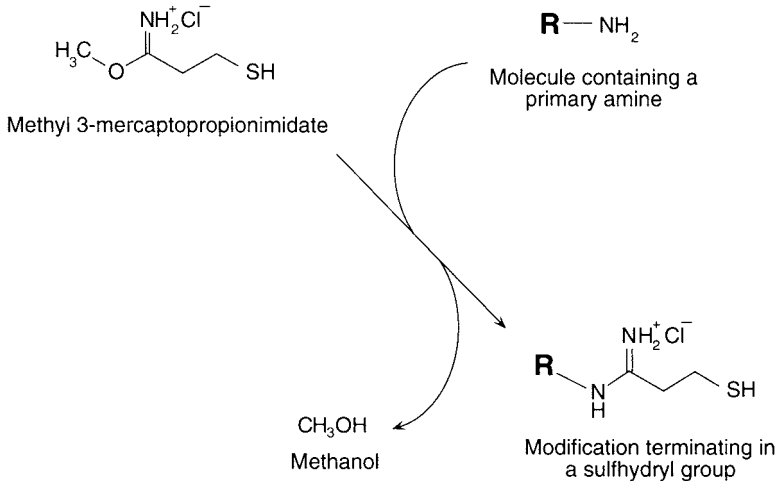
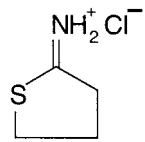


Figure 1.59 Thiolation of an amine-containing compound with methyl 3-mercaptopropionimidate. The modification preserves the positive charge on the primary amine.

imidoester, forming 2-iminothiolane (Jue *et al.*, 1978). The cyclic imidothioester still can react with primary amines in a ring-opening reaction that regenerates the free sulfhydryl (Figure 1.60).



Traut's Reagent;
2-Iminothiolane
MW 137.6
8.1 Å

Traut's reagent is fully water-soluble and reacts with primary amines in the range of pH 7–10. The cyclic imidothioester is stable to hydrolysis at acid pH values, but its half-life in solution decreases as the pH increases beyond neutrality. However, even at pH 8.0 in 25 mM triethanolamine the rate of sulfhydryl formation without added primary amine was found to be negligible. Upon addition of dipeptide amine, the reagent reacted quickly as evidenced by the production of Ellman's reagent color. The rate of reaction also can be followed by 2-iminothiolane's absorbance at 248 nm (λ_{max}; $\varepsilon = 8{,}840\,M^{-1}cm^{-1}$). As the cyclic imidate reacts with amines, its absorbance at this wavelength decreases. With addition of the dipeptide glycylglycine, the starting absorbance of a solution of Traut's reagent decreased over 80 percent within 20 minutes (Jue *et al.*, 1978). Thus, protein modification with 2-iminothiolane is very efficient and proceeds rapidly at slightly basic pH.

At high pH (10.0), Traut's reagent also is reactive with aliphatic and aromatic hydroxyl groups, although the rate of reaction with these groups is only about 0.01 that of primary

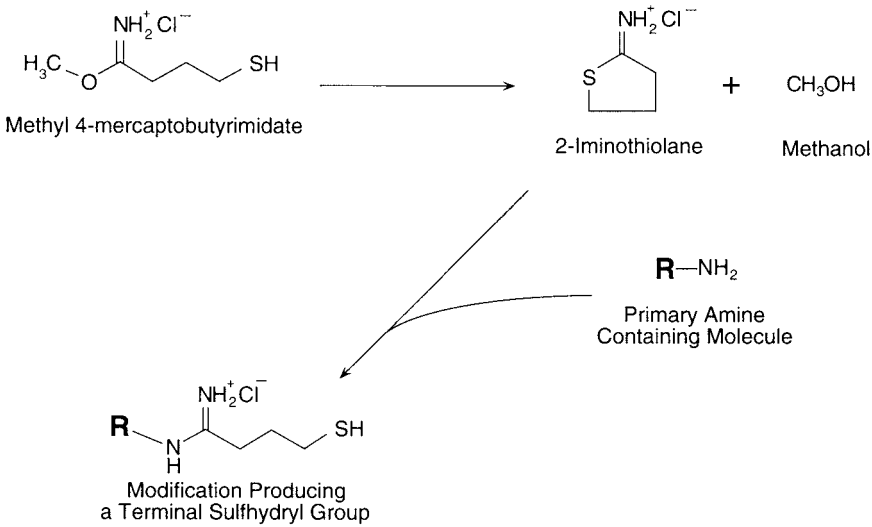


Figure 1.60 Methyl 4-mercaptobutyrimidate forms 2-iminothiolane, which can react with a primary amine to create a sulfhydryl group. The modification preserves the positive charge of the original amine.

amines. In the absence of amines, however, carbohydrates such as agarose or cellulose membranes can be modified to contain sulfhydryl residues (Alagon and King, 1980). Polysaccharides modified in this manner are effective in covalently crosslinking antibodies for use in immunoassay procedures.

Proteins modified with 2-iminothiolane are subject to disulfide formation upon sulfhydryl oxidation. This can cause unwanted conjugation, potentially precipitating the protein. The addition of a metal-chelating agent such as EDTA (0.01–0.1 M) will prevent metal-catalyzed oxidation and maintain sulfhydryl stability. In the presence of some serum proteins (i.e., BSA) a 0.1 M concentration of EDTA may be necessary to prevent metal-catalyzed oxidation, presumably due to the high contamination of iron from hemolyzed blood.

Traut's reagent has been used successfully in the investigation of ribosomal proteins (Sun *et al.*, 1974; Jue *et al.*, 1978; Kenny *et al.*, 1979; Lambert *et al.*, 1983; Blattler *et al.*, 1985b), RNA polymerase (Hillel and Wu, 1977), progesterone receptor subunits (Birnbaumer *et al.*, 1979), and in the synthesis of enzyme-labeled DNA hybridization probes (Ghosh *et al.*, 1990). It is an excellent thiolation reagent for use in the preparation of immunotoxins (Section 3.3). It also has been used to modify and introduce sulfhydryls into oligosaccharides from asparagine-linked glycans (Tarentino *et al.*, 1993).

Side reactions other than oxidation to disulfides also can occur using Traut's reagent. Once an amine on a protein is modified with 2-iminothiolane, the terminal thiol can recyclize by attacking the amidine carbon (Figure 1.61). This then can rearrange into an iminothiolane derivative, which effectively ties up the thiol (Singh *et al.*, 1996; Mokotoff *et al.*, 2001). Proteins and other molecules thiolated using Traut's reagent can loose substantial amounts of available thiol to recyclization in just hours. For this reason, the thiolated product of a Traut's reaction should be used immediately in a conjugation reaction to avoid significant loss of activity.

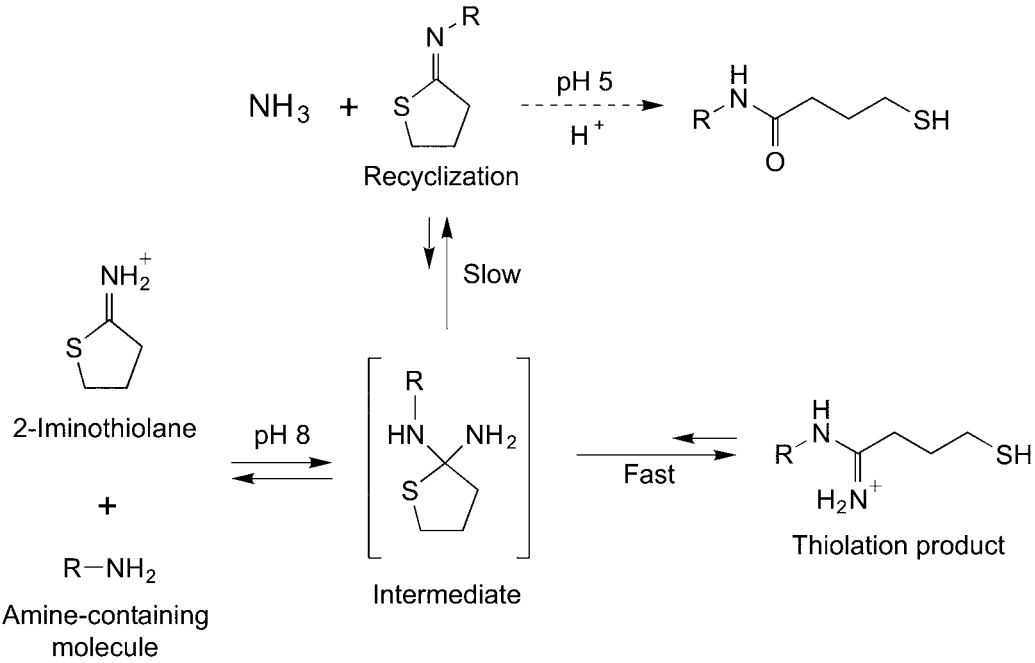


Figure 1.61 Traut's reagent can undergo side reactions after the modification of an amine-containing molecule. The terminal thiol group can recyclize to create another iminothiolane derivative that effectively ties up the thiol.

Protocol

1. Prepare the protein or macromolecule to be thiolated in a non-amine containing buffer at pH 8.0. For the modification of ribosomal proteins (often cited in the literature) use 50 mM triethanolamine hydrochloride, 1 mM $MgCl_2$, 50 mM KCl, pH 8.0. The magnesium and potassium salts are for stabilization of some ribosomal proteins. If other proteins are to be thiolated, the same buffer may be used without added salts for stabilization. Alternatively, 50 mM sodium phosphate, 0.15 M NaCl, pH 8.0, or 0.1 M sodium borate, pH 8.0 may be used. For the modification of polysaccharides, use 20 mM sodium borax, pH 10, to produce reactivity toward carbohydrate hydroxyl residues. Dissolve the protein to be modified at a concentration of 10 mg/ml in the reaction buffer of choice. Lower concentrations also may be used with a proportional scaling back of added 2-iminothiolane.
2. Dissolve the Traut's reagent (Thermo Fisher) in water at a concentration of 2 mg/ml (makes a 14.5 mM stock solution). The solution should be used immediately. For the modification of IgG at a concentration of 10 mg/ml using a 10-fold molar excess of Traut's reagent, add 45.8 µl of the stock solution to each ml of the protein solution.
3. React for 1 hour at room temperature (a 4°C reaction temperature may be used successfully as well).
4. Purify the thiolated protein from unreacted Traut's reagent by gel filtration using your buffer of choice (i.e., 20 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.2). The addition of EDTA to this buffer helps to prevent oxidation of the sulfhydryl groups and the resultant disulfide formation. After purification, use the thiolated protein immediately

in a conjugation reaction to avoid the recyclization of the free thiol with concomitant decrease in thiol availability.
5. The degree of —SH modification may be determined using the Ellman's assay (Section 4.1, this chapter).

When 2-iminothiolane is used to modify proteins in tandem with 4,4′-dipyridyl disulfide, a protected sulfhydryl can be introduced in a single step (King *et al.*, 1978). The simultaneous reaction between a protein, 2-iminothiolane and 4,4′-dipyridyl disulfide yields a modification containing pyridyl disulfide groups. The pyridyl disulfide subsequently may be reduced with dithiothreitol (DTT) to yield a free sulfhydryl. Pyridyl disulfides also are highly reactive toward sulfhydryls through disulfide interchange (Chapter 2, Section 2.6). The protocol is a modification of the method of King *et al.*, 1978.

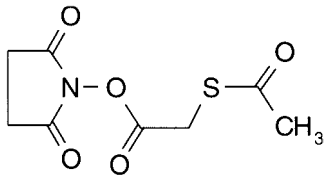
Protocol

1. Dissolve 1–10 mg of a protein to be modified in 1.0 ml of 0.025 M sodium borate, pH 9.0.
2. Dissolve 2-iminothiolane in 0.025 M sodium borate to a concentration of 0.02 M.
3. Dissolve 4,4′-dipyridyl disulfide at a concentration of 2 mg/ml in acetonitrile.
4. Add 0.2 ml of (3) and 1.0 ml of (2) to the protein solution.
5. React for 2 hours at room temperature.
6. Purify the modified protein by gel filtration or dialysis.

Occasionally, a protein modified in this manner will begin to precipitate as the reaction proceeds. Stopping the reaction earlier or adding a smaller quantity of modifying reagents may limit this effect.

Modification of Amines with SATA

A versatile reagent for introducing sulfhydryl groups into proteins is SATA, *N*-succinimidyl *S*-acetylthioacetate (Duncan *et al.*, 1983). The active NHS ester end of SATA reacts with amino groups in proteins and other molecules to form a stable amide linkage (Figure 1.62) (Chapter 2, Section 1.4). The modified protein then contains a protected sulfhydryl that can be stored without degradation and subsequently deprotected as needed with an excess of hydroxylamine (Figure 1.63). Since the protecting group can be removed without adding disulfide reducing agents like DTT, disulfides indigenous to the native protein will not be affected. This is an important consideration if disulfides are vital to activity, such as in the case of antibodies and some protein toxins.



SATA;
N-succinimidyl
S-acetylthioacetate
MW 231.2

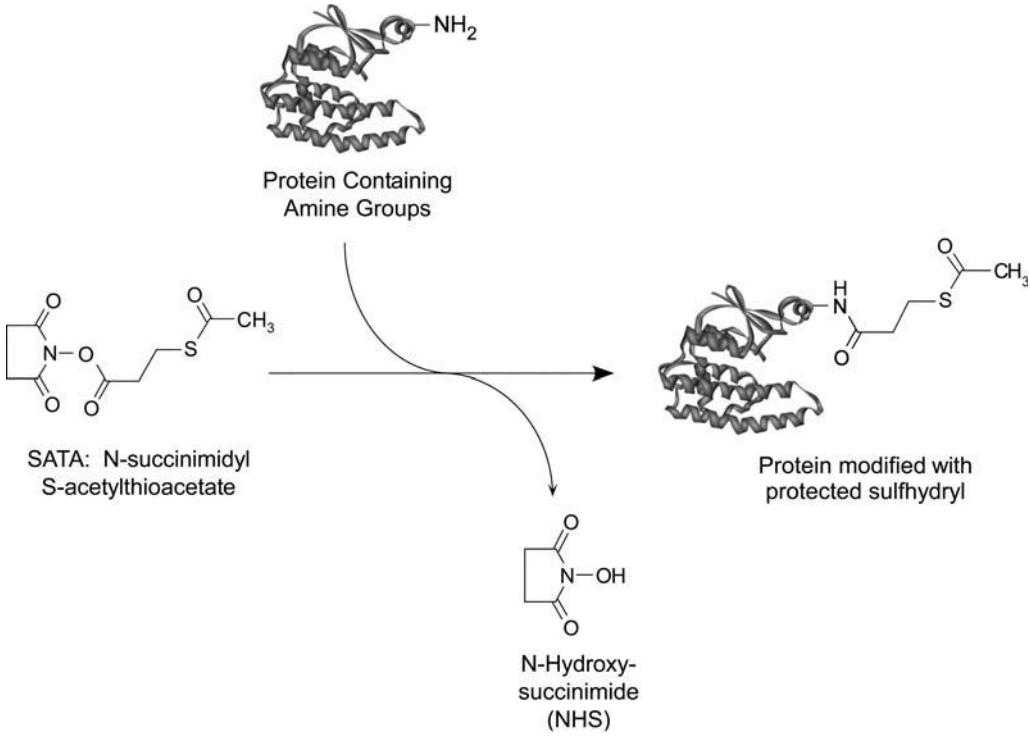


Figure 1.62 SATA can react with available amine groups in proteins and other molecules via its NHS ester end to form protected sulfhydryl derivatives. The illustrated protein is glutathione-*S*-transferase (E.C.2.5.1.18) (Ji *et al*., 1995).

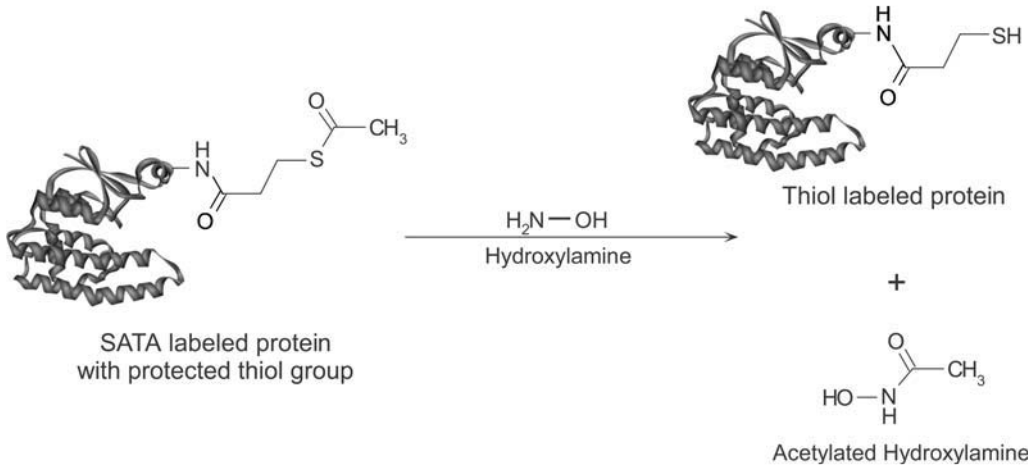


Figure 1.63 Deprotection with hydroxylamine of the acetylated thiol of SATA-modified proteins yields a free sulfhydryl group.

SATA is often used to form antibody–enzyme conjugates utilizing maleimide-containing heterobifunctional crosslinking agents. Most polyclonal antibody molecules may be modified to contain up to about 6 SATA molecules per immunoglobulin with minimal effect on antigen binding activity. Some sensitive monoclonal antibodies, however, may be susceptible to modification and should be tested on a case-by-case basis. The modified antibody then may be deprotected and reacted with a maleimide-activated enzyme to form a conjugate useful in immunoassays (Chapter 20, Section 1.1). Conjugates formed using SATA are usually of low molecular weight with very few high-molecular-weight oligomers. They also maintain a bivalent antibody structure, assuring a conjugate containing two antigen binding sites. This is an advantage over reduction schemes that break the antibody molecule into two heavy–light chain pairs to create sulfhydryls, since disulfide cleavage yields antibody fragments with only one antigen binding site.

SATA has been used to form conjugates with avidin or steptavidin with excellent retention of activity (Chapter 23, Section 3.1). It also has been used in the formation of a therapeutically useful toxin conjugate with recombinant CD4 (Ghetie *et al*., 1990), to study syntaxin proteins (Amessou *et al*., 2007), to prepare bispecific antibodies (Lindorfer *et al*., 2001), and to make a unique polylysine conjugate as a vehicle for drug delivery (Sakharov *et al*., 2001).

SATA is freely soluble in many organic solvents. In use, it is typically dissolved as a stock solution in DMSO, DMF, or methylene chloride, and then an aliquot of this solution is added to an aqueous reaction mixture containing the protein to be modified.

The thiolation method described below is generally applicable for the modification of proteins with SATA, particularly for subsequent conjugation with a maleimide-activated secondary protein. The degree of modification described usually yields 3–4 moles of —SH groups per mole protein when thiolating immunoglobulins. Other macromolecules containing primary amines may be modified using a similar procedure. The degree of modification observed with other molecules may vary depending on the number of available primary amines and their relative reactivity. The molar ratio of SATA to immunoglobulin added to a reaction for the modification of rabbit polyclonal IgG versus the degree of sulfhydryl incorporation is illustrated in Figure 1.64 (Sykaluk, 1994).

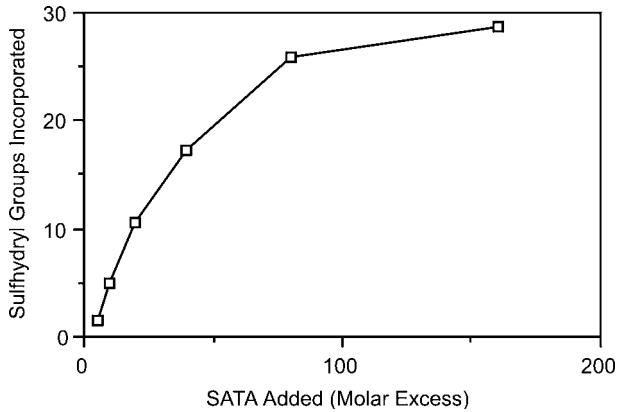


Figure 1.64 SATA modification of rabbit polyclonal IgG with the resultant sulfhydryl incorporation level.

The following protocol represents a generalized method for protein thiolation using SATA. For comparison purposes, contrast the variation of this SATA modification method as outlined in Chapter 20, Section 1.1 for use in the preparation of antibody–enzyme conjugates.

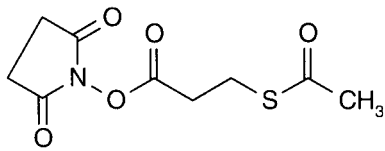
Protocol

1. Dissolve the protein to be thiolated at a concentration of 1–5 mg/ml in 50 mM sodium phosphate, pH 7.5, containing 1–10 mM EDTA. Other non-amine containing buffers such as borate, HEPES, and bicarbonate also may be used as the reaction medium. The effective pH for the NHS ester modification reaction is in the range of 7.0–9.0, but environments closer to neutrality will limit the hydrolysis of the ester.
2. Dissolve the SATA reagent (Thermo Fisher) in DMSO at a concentration of 65 mM (15 mg/ml). *Note*: DMSO should be handled in a fume hood.
3. Add 10 µl of the SATA solution to each ml of protein solution.
4. Mix and react for 30 minutes at room temperature.
5. Separate modified protein from unreacted SATA and reaction by-products by dialysis against 50 mM sodium phosphate, pH 7.5, containing 1 mM EDTA or by gel filtration on a Sephadex G-25 column (Pharmacia) using the same buffer.
6. Deprotect the acetylated —SH groups as needed by adding 100 µl of 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5, to each ml of the SATA-modified protein solution.
7. Mix and react for 2 hours at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 mM sodium phosphate, 1 mM EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (Section 4.1, this chapter).

Modification of Amines with SATP

SATP, succinimidyl acetylthiopropionate, is an analog of SATA (Chapter 1, Section 4.1) containing one additional carbon atom in length (Fuji *et al*., 1985). The compound retains all the advantages of a protected sulfhydryl, including stability of the modified protein and selective release of the protecting group with hydroxylamine to free the sulfhydryl as needed (Figure 1.65). SATP is soluble in DMF and methylene chloride. It is usually first solubilized in organic solvent and an aliquot added to an aqueous solution containing the macromolecule to be modified. It is particularly useful in adding an N-terminal —SH group at the completion of peptide synthesis.



SATP
Succinimidyl acetyl-
thiopropionate
MW 245

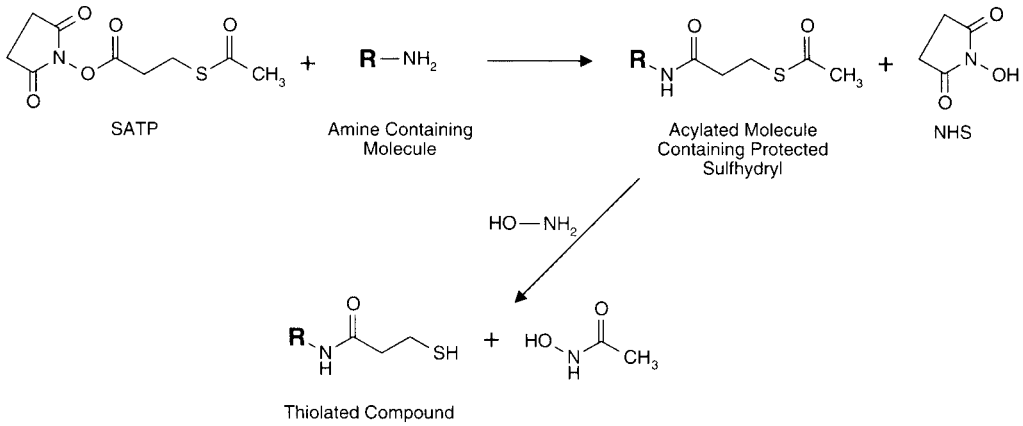


Figure 1.65 SATP reacts with amine-containing proteins or other molecules via its NHS ester end to create protected sulfhydryl derivatives in a manner similar to that of SATA. Deprotection can be done with hydroxylamine to free the thiol.

Protocol

1. Dissolve the protein or peptide to be thiolated at a concentration of 10 mg/ml in 50 mM sodium phosphate, pH 7.5, containing 1 mM EDTA. Other non-amine containing buffers such as borate, HEPES, and bicarbonate also may be used as the reaction medium. The effective pH for the NHS ester modification reaction is in the range of 7.0–9.0. Conditions closer to neutral pH will limit the degree of NHS ester hydrolysis during the reaction.
2. Dissolve the SATP reagent (Molecular Probes) in DMF at a concentration of 65 mM (16 mg/ml). *Note*: DMF should be handled in a fume hood.
3. Add 10 µl of the SATP solution to each ml of protein or peptide solution.
4. Mix and react for 30–60 minutes at room temperature (or 2–4 hours at 4°C).
5. Separate modified protein from unreacted SATP and reaction by-products by dialysis against 50 mM sodium phosphate, pH 7.5, containing 1 mM EDTA or by gel filtration using a desalting column with the same buffer. If a peptide of low molecular weight is being modified, careful gel filtration using a matrix having a low exclusion limit will separate the peptide from the reaction by-products, but the separation should be done on an automated system to accurately capture the peaks. In this case, use either Sephadex G-25 or Sephadex G-10 for the chromatography.
6. Deprotect the acetylated —SH groups as needed by adding 100 µl of 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5, to each ml of the SATP-modified protein solution.
7. Mix and react for 2 hours at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 mM sodium phosphate, 1 mM EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer. Again, if a peptide of low molecular weight is being modified, use careful gel filtration for purification.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (Section 4.1, this chapter).

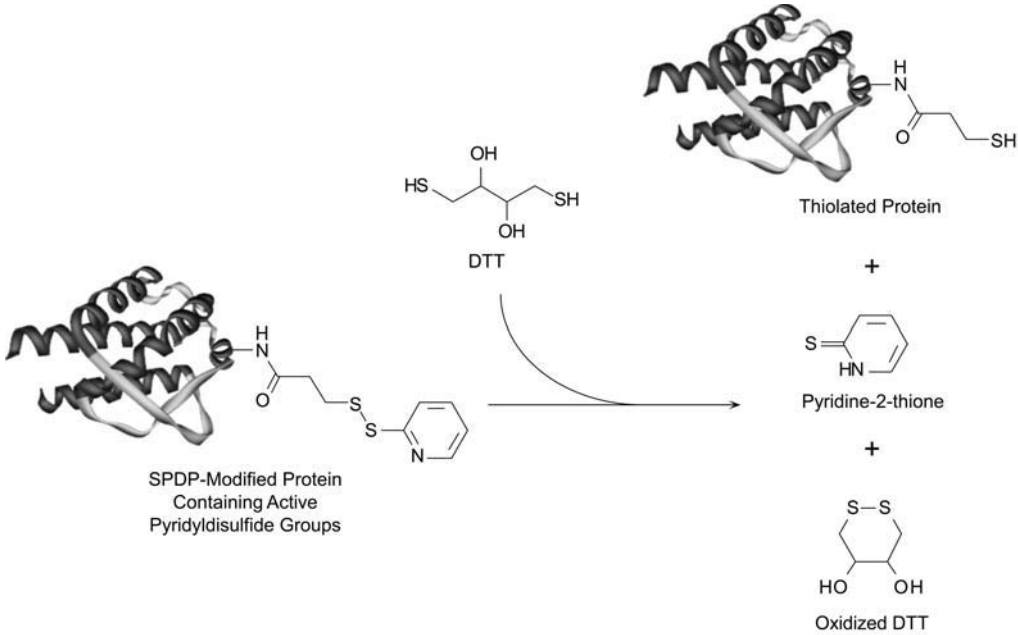


Figure 1.66 SPDP-modified proteins can be reduced with DTT to yield free sulfhydryl groups for conjugation.

Modification of Amines with SPDP

SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate, is one of the most popular heterobifunctional crosslinking agents (Chapter 5, Section 1.1). The NHS ester end of SPDP reacts with amine groups to form an amide linkage, while the 2-pyridyldithiol group at the other end can react with sulfhydryl residues to form a disulfide linkage (Carlsson *et al.*, 1978). The crosslinker is used extensively to form immunotoxin conjugates for *in vivo* administration (Chapter 21, Section 2.1). The reagent is also useful in creating sulfhydryls in proteins and other molecules. Once modified with SPDP, a protein can be treated with DTT (or other disulfide reducing agents, see Section 4.1, this chapter) to release the pyridine-2-thione leaving group and form the free sulfhydryl (Figure 1.66). The terminal —SH group then can be used to conjugate with any crosslinking agents containing sulfhydryl-reactive groups, such as maleimide or iodoacetyl functionalities (for covalent conjugation) or 2-pyridyldithiol groups (for reversible conjugation).

There are three forms of SPDP analogs currently available commercially (Thermo Fisher): the standard SPDP, a long-chain version designated LC-SPDP, and a water-soluble, sulfo-NHS form also containing an extended chain, called sulfo-LC-SPDP (Chapter 5, Section 1.1). The main disadvantage to using SPDP to create sulfhydryls is the necessity of using a reducing agent to remove the pyridine-2-thione group. Reducing agents also will affect indigenous disulfides within a protein molecule, cleaving and reducing them. This method therefore works well for proteins containing no sulfhydryls or no disulfides that are critical to function, but it may cause loss of activity or subunit breakdown in proteins containing essential disulfides.

The following procedure is similar to the method of Cumber *et al.* (1985), but with some modifications.

Protocol

1. Dissolve the protein or macromolecule to be thiolated at a concentration of 10 mg/ml in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Other non-amine containing buffers such as borate, HEPES, and bicarbonate also may be used in this reaction. The effective pH for the NHS ester modification reaction is in the range of 7.0–9.0, but environments closer to neutrality will limit ester hydrolysis.
2. Dissolve SPDP (Thermo Fisher) at a concentration of 6.2 mg/ml in DMSO (makes a 20 mM stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 mM solution). If the water-soluble sulfo-LC-SPDP is used for the reaction, a stock solution in water may be prepared just prior to adding an aliquot to the protein solution. In this case, prepare a 10 mM solution of sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified to allow accurate weighing of sulfo-LC-SPDP, the solid may be added directly to the reaction mixture without preparing a stock solution in water.
3. Add 25 µl of the stock solution of either SPDP or LC-SPDP in DMSO to each ml of the protein to be modified. If sulfo-LC-SPDP is used, add 50 µl of the stock solution in water to each ml of protein solution.
4. Mix and react for at least 30 minutes at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2.
6. To release the pyridine-2-thione leaving group and form the free sulfhydryl, add DTT at a concentration of 0.5 mg DTT per mg of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per ml of 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5. Add 25 µl of this solution per mg of modified protein. Release of pyridine-2-thione can be followed by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3\,M^{-1}cm^{-1}$).
7. Mix and react at room temperature for 30 minutes.
8. Purify the thiolated protein from excess DTT by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.2. The modified protein should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide crosslinks.

Modification of Amines with SMPT

SMPT, succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene, contains an NHS ester end and a pyridyl disulfide end similar to SPDP, but its hindered disulfide makes conjugates formed with this reagent more stable (Thorpe *et al.*, 1987) (Chapter 5, Section 1.2). The reagent is especially useful in forming immunotoxin conjugates for *in vivo* administration (Chapter 21, Section 2.1). A water-soluble analog of this crosslinker containing an extended spacer arm is also commercially available as sulfo-LC-SMPT (Thermo Fisher).

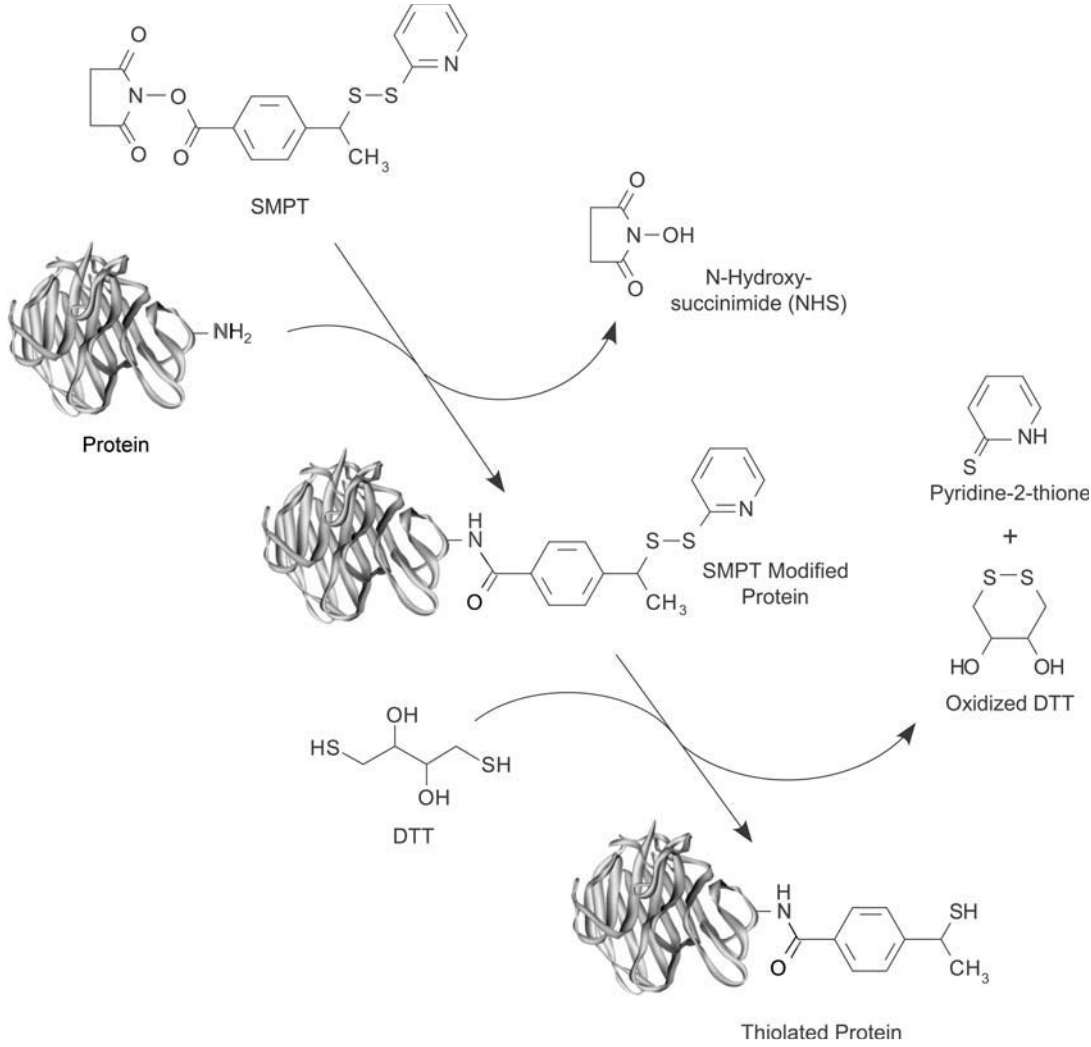


Figure 1.67 SMPT can be used to modify the amine groups of proteins to form disulfide intermediates. The disulfides can be reduced with DTT to create free thiols for subsequent conjugation purposes.

SMPT or sulfo-LC-SMPT may be used as thiolation reagents by first reacting its NHS ester end with an amine-containing molecule and then releasing the pyridine-2-thione leaving group with DTT to free the sulfhydryl (Figure 1.67). The disadvantage of this approach is the necessity of using a reducing agent to create the —SH group modification. This method of thiolation only should be used if there are no disulfides in the target molecule that are critical to function. If a reductant cannot be used, choose a thiolation method that does not need DTT treatment, such as the use of Traut's reagent or SATA (Section 4.1, this chapter).

Since SMPT is not soluble in aqueous solutions it must be first dissolved in organic solvent and an aliquot of this stock solution transferred to the reaction solution. The reagent is soluble

in DMF and DMSO, but is much more stable in solutions of acetonitrile. A stock solution of SMPT in acetonitrile may be kept frozen without loss of activity. The NHS ester of SMPT also is extraordinarily stable to hydrolysis in water. Even when an SMPT/acetonitrile aliquot is added to an aqueous solution and stored at room temperature, SMPT will only lose about 5 percent of its activity after 16 hours. By contrast, other NHS esters usually have half-lives measured in minutes or hours in aqueous environments, depending on the pH.

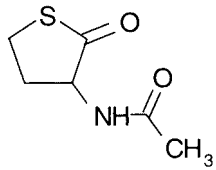
Sulfo-LC-SMPT is not as stable as SMPT. The sulfo-NHS ester is more susceptible to hydrolysis in aqueous solutions and the pyridyl disulfide group is more easily reduced to the free sulfhydryl. Stock solutions of sulfo-LC-SMPT may be prepared in water, but should be used immediately to prevent loss of amine coupling ability.

Protocol

1. Dissolve the protein or macromolecule to be thiolated at a concentration of 10 mg/ml in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Other non-amine-containing buffers such as borate, HEPES, and bicarbonate also may be used as the reaction medium. The effective pH for the NHS ester modification reaction is in the range of 7.0–9.0, but conditions close to neutrality will limit ester hydrolysis.
2. Dissolve SMPT (Thermo Fisher) at a concentration of 7.7 mg/ml in acetonitrile (makes a 20 mM stock solution). Alternatively, the water-soluble sulfo-LC-SMPT may be used and dissolved at a concentration of 6.0 mg/ml in water (makes a 10 mM solution). This should be done just prior to adding an aliquot to the thiolation reaction. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified to allow accurate weighing of sulfo-LC-SMPT, the solid may be added directly to the reaction mixture without preparing a stock solution in water, but this is not recommended with most reactions.
3. Add 25 µl of the stock solution of SMPT in acetonitrile to each ml of the protein to be modified. If sulfo-LC-SMPT is used, add 50 µl of the stock solution in water to each ml of protein solution.
4. Mix and react for at least 30 minutes at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2.
6. To release the pyridine-2-thione leaving group and form the free sulfhydryl, add DTT at a concentration of 0.5 mg DTT per mg of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per ml of 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5. Add 25 µl of this solution per mg of modified protein. Release of pyridine-2-thione can be followed by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3 M^{-1}cm^{-1}$).
7. Mix and react at room temperature for 30 minutes.
8. Purify the thiolated protein from excess DTT by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.2. The modified protein should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide crosslinks.

Modification of Amines with N-Acetyl Homocysteine Thiolactone

N-Acetyl homocysteine thiolactone (also called citiolone or 2-acetamido-4-mercaptobutyric acid) is a cyclic derivative of homocysteine containing a blocked α-amino group. The compound can react with primary amines in a ring-opening reaction to create free sulfhydryl modifications (Figure 1.68). It was originally used as a reagent for insolubilizing antibodies. Later, it was immobilized on an amine-containing matrix to form a disulfide reducing support for cleaving cystine residues in peptides and proteins (Eldjarn and Jellum, 1963; Jellum, 1964) (see Use of Disulfide Reductants, this section). The thiolation reaction of amine-containing macromolecules proceeds much like the reaction for 2-iminothiolane. Nucleophilic attack occurs at the carbonyl, cleaving the thiolactone and producing an amide linkage with the target molecule, while at the same time creating the free sulfhydryl (Benesch and Benesch, 1956, 1958). *N*-Acetyl homocysteine is soluble in aqueous buffers.



N-Acetyl Homocysteine
Thiolactone
MW 159

Thiolation of peptides and other small molecules containing amines proceeds easily with *N*-acetyl homocysteine thiolactone. However, protein modification often results in much lower yields unless the reaction is done for extended periods at pH 10–11.

It has been found that silver ions catalyze the thiolation process with proteins, allowing the reaction to be completed rapidly at physiological pH (Benesch and Benesch, 1958). The addition of an equal molar concentration of $AgNO_3$ forms an insoluble complex with the thiolactone, and this in turn reacts with protein amines.

Protocol

1. Dissolve the amine-containing molecule to be thiolated at a concentration of 10 mg/ml in cold (4°C) 1 M sodium bicarbonate (reaction buffer). For proteins, dissolve them in deionized water at a pH of 7.0–7.5, at room temperature. *Note*: The presence of some buffer salts, like phosphate or carbonate, is incompatible with silver nitrate.
2. Add *N*-acetyl homocysteine thiolactone (Aldrich) to the bicarbonate reaction mixture to obtain a concentration representing a 10- to 20-fold excess over the amount of amines present. For protein thiolation, add the same molar excess of thiolactone reagent to the water reaction medium, and then slowly add an equivalent molar quantity of silver nitrate ($AgNO_3$). Maintain the pH at 7.0–7.5 with periodic addition of NaOH.
3. For the bicarbonate reaction, gently mix for 20 hours at 4°C. For the silver-catalyzed reaction, continue the reaction for 1 hour or until the silver complex has fully dissolved.

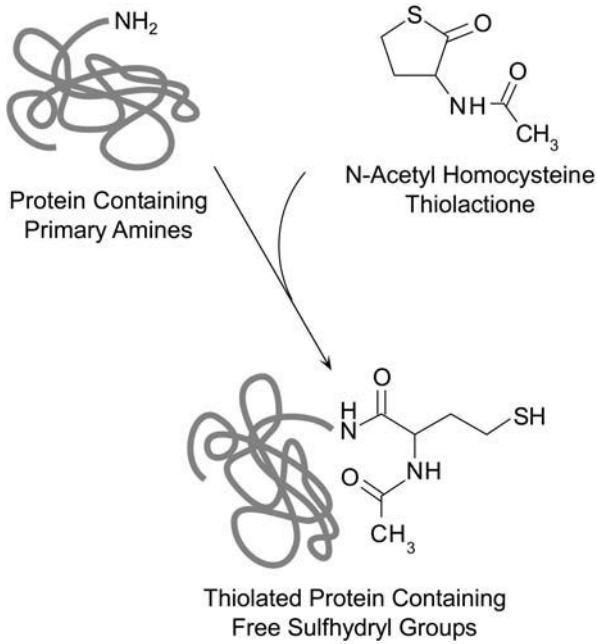


Figure 1.68 *N*-Acetyl homocysteine thiolactone spontaneously reacts with amine groups on proteins to create sulfhydryl groups.

4. To remove the silver mercaptide formed from the facilitated protein thiolation reaction, add an excess of thiourea to convert all the silver into a soluble $Ag(thiourea)_2^+$ complex and free the sulfhydryl modifications.
5. Remove unreacted *N*-acetyl homocysteine thiolactone and reaction by-products by gel filtration or dialysis against 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. Other buffers suitable for individual protein stability may be used as desired. For the silver nitrate-containing reaction, removal of the silver–thiourea complex may be done by adsorption onto Dowex 50, and the protein subsequently eluted from the resin by 1 M thiourea. Removal of the thiourea then may be done by gel filtration or dialysis.

Including EDTA in the final preparation inhibits metal-catalyzed oxidation of the sulfhydryl groups to disulfides. The modified peptide or protein should be used immediately to assure full sulfhydryl reactivity.

Modification of Amines with SAMSA

S-Acetylmercaptosuccinic anhydride, or SAMSA, is an amine-reactive reagent containing a protected sulfhydryl much like SATA described previously. The anhydride portion opens in response to the attack of an amine nucleophile, yielding an amide linkage (Klotz and Heiney, 1962; Weston *et al.*, 1980). The ring-opening reaction, however, does produce a free carboxylate group that lends a negative charge to the modified molecule where once there may have been a positive charge (Figure 1.69). This charge reversal may affect the conformation and

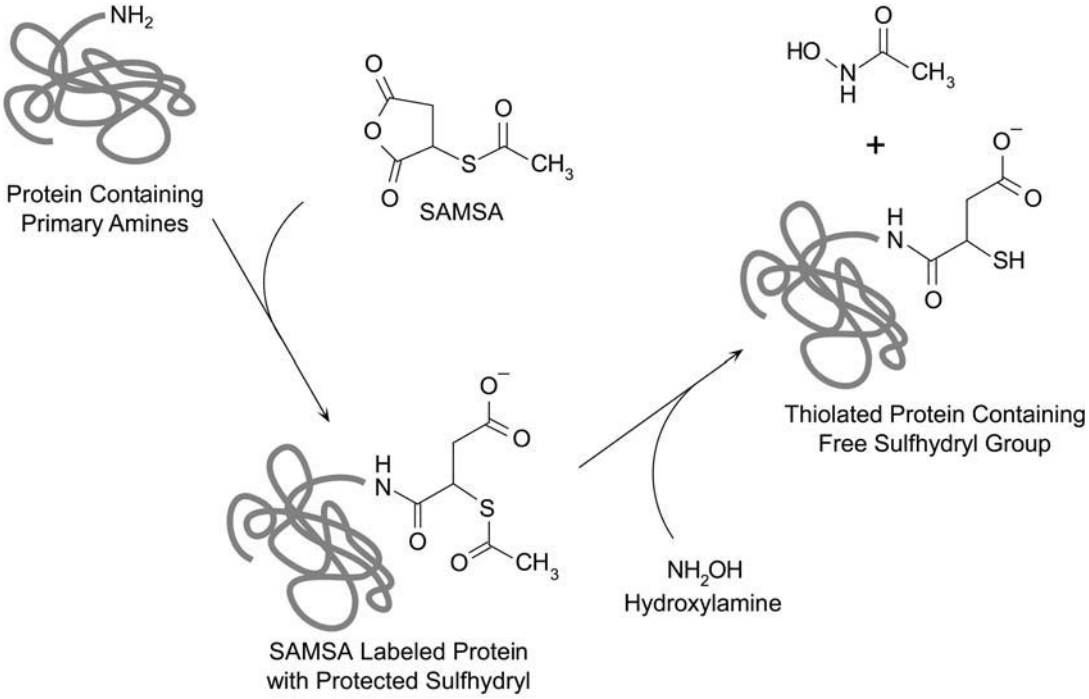
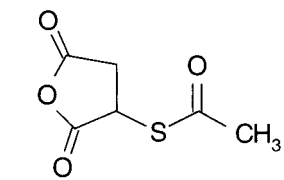


Figure 1.69 SAMSA is an anhydride compound containing a protected thiol. Reaction with protein amine groups yields amide bond linkages. Deprotection of the acetylated thiol produces free sulfhydryl groups for conjugation.

activity of some sensitive proteins. After the initial modification step, releasing the acetylated sulfhydryl-protecting group with hydroxylamine forms the thiolated derivative.



SAMSA;
S-Acetylmercaptosuccinic
Anhydride
MW 174

Protocol

1. Dissolve the protein or other amine-containing macromolecule in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 5 mg/ml.
2. Dissolve SAMSA in DMF at a concentration of 25 mg/ml.
3. Add 20 µl of the stock SAMSA solution to each ml of the protein solution, with mixing.
4. React at room temperature for 30 minutes.

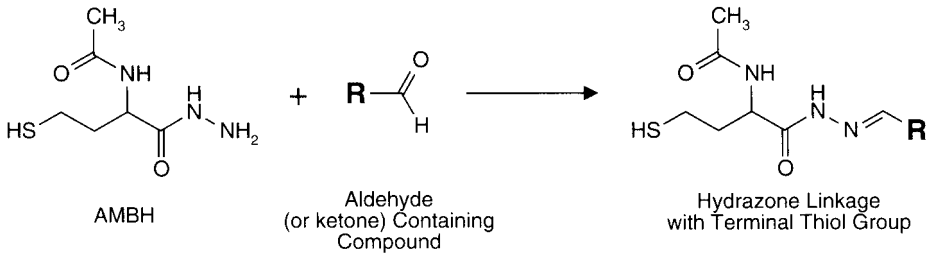


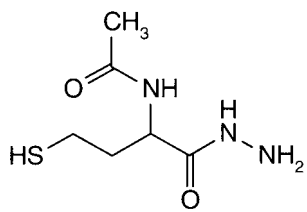
Figure 1.70 AMBH is a hydrazide-containing compound that reacts with carbonyl groups to form hydrazone bonds. The free thiol can be used for subsequent conjugation reactions.

5. Remove excess reagent and reaction by-products by dialysis or gel filtration using 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5. For chromatographic separation, use a desalting gel filtration support such as the Zeba desalting spin columns (Thermo Fisher) or the equivalent. The SAMSA-modified protein may be stored at −20°C until needed.
6. To deprotect the acetylated sulfhydryl group of SAMSA-modified proteins, add 100 µl of 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5, to each ml of protein solution.
7. Mix and react for 2 hours at room temperature.
8. Purify the sulfhydryl-modified protein by dialysis against 50 mM sodium phosphate, 1 mM EDTA, pH 7.5, or by gel filtration on a Sephadex G-25 column using the same buffer.

The deacetylated protein should be used immediately to prevent loss of sulfhydryl content through disulfide formation. The degree of —SH modification may be determined by performing an Ellman's assay (see Ellman's Assay for the Determination of Sulfhydryls, this chapter).

Modification of Aldehydes or Ketones with AMBH

AMBH (2-acetamido-4-mercaptobutyric acid hydrazide) is a unique hydrazide derivative that can thiolate aldehydes and ketones to form reactive sulfhydryl groups (Taylor and Wu, 1980). It is particularly useful in converting oxidized carbohydrates to contain a thiol. In this respect, glycoproteins or other carbohydrate and diol-containing molecules may be treated with sodium periodate under relatively mild conditions to form aldehyde residues (Section 4.4, this chapter). The aldehydes readily react with the hydrazide groups of AMBH to form hydrazone linkages, leaving a free terminal sulfhydryl residue to use in further conjugation reactions (Figure 1.70).



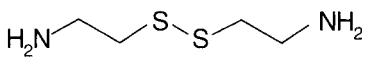
AMBH
2-Acetamido-4-mercaptobutyric
acid hydrazide
MW 191

Protocol

1. Dissolve an aldehyde-containing macromolecule to be modified (i.e., a periodate-oxidized glycoprotein) in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4, containing 1 mM EDTA. A suitable concentration range for a protein is 1–10 mg/ml.
2. Add a 10-fold molar excess of AMBH (pre-dissolved in ethanol) (Molecular Probes) over the expected amounts of aldehydes to be modified.
3. React for 2 hours at room temperature.
4. Purify the modified protein by gel filtration.

Modification of Carboxylates or Phosphates with Cystamine

Cystamine is decarboxylated cystine [or 2,2′-dithio*bis*(ethylamine)], a small disulfide-containing molecule with primary amines at both ends. This versatile reagent can be used in several conjugation techniques. Cystamine may be used to introduce sulfhydryl residues in proteins, nucleic acids, and other molecules, or as the active species in disulfide exchange crosslinking reactions, or in reversible conjugation procedures. The reagent can be used to create sulfhydryl groups in proteins or other molecules by first conjugating one of its terminal amino groups with the carboxylates on a target molecule using a carbodiimide reaction (Chapter 2, Section 1.11 and Chapter 3, Section 1). Subsequent reduction of the disulfide group liberates the free sulfhydryl (see Ellman's Assay for the Determination of Sulfhydryls, this section) (Figure 1.71). This same modification procedure also can be used to introduce sulfhydryl residues at the 5′-phosphate group of DNA (Chu *et al.*, 1986; Ghosh *et al.*, 1990). The carbodiimide activates the phosphate and the amines of cystamine may then react with this active species to form a phosphoramidate bond (Chapter 27, Section 2.2) (Figure 1.72). Specific labeling of DNA probes only at the 5′-end is possible using this technique.



Cystamine;
2,2'-dithiobis(ethylamine)
MW 152

The carbodiimide of choice used to couple cystamine to carboxylate- or phosphate-containing molecules is most often the water-soluble carbodiimide, EDC hydrochloride; Chapter 3, Section 1.1). This reagent rapidly reacts with carboxylates or phosphates to form an active ester intermediate, which is highly reactive toward primary amines. The reaction is efficient from pH 4.7 to 7.5, and a variety of buffers may be used, providing they don't contain competing groups.

Cystamine also is used as an activating reagent for disulfide exchange reactions. In this procedure, the reagent is used to modify one of two proteins to be conjugated. The cystamine-modified protein then is mixed with the other protein that contains, or is thiolated to contain, a sulfhydryl group. By disulfide exchange, the sulfhydryl-containing molecule cleaves the disulfide of the cystamine-modified protein, releasing 2-mercaptoethylamine and forming a disulfide crosslink (Figure 1.73).

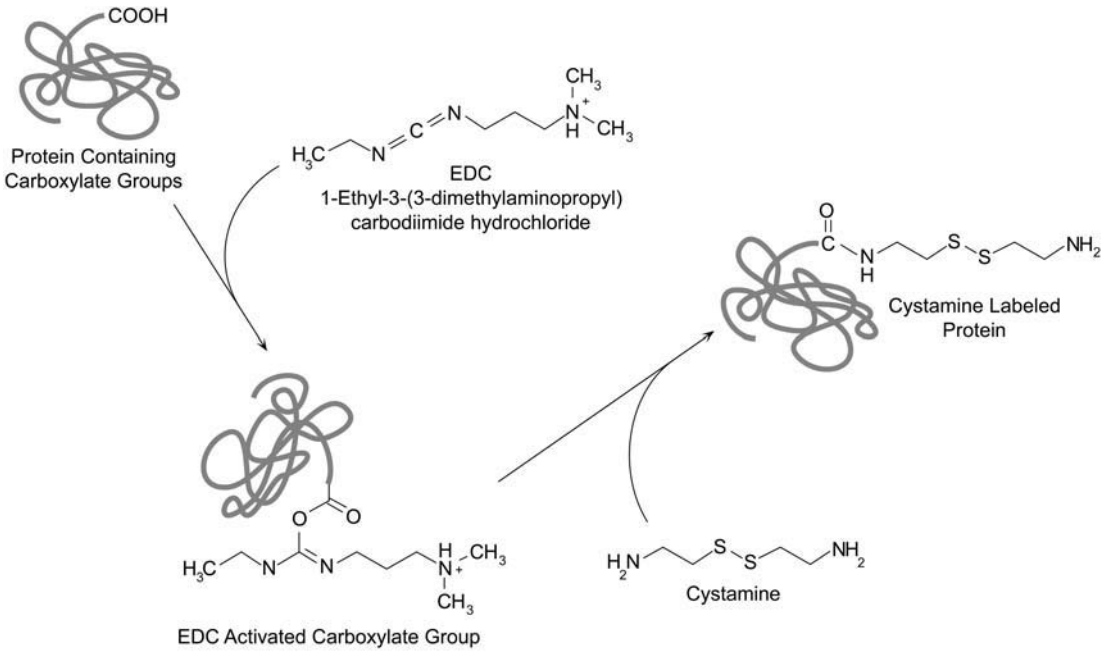


Figure 1.71 Cystamine may be used to label protein carboxylate groups using the water-soluble carbodiimide EDC.

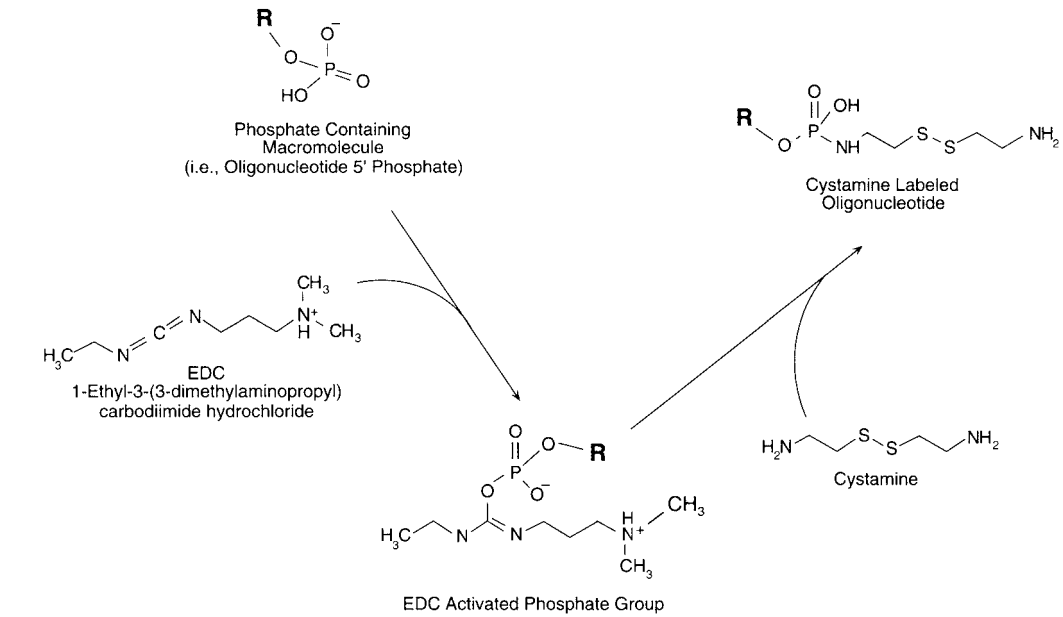


Figure 1.72 Cystamine may be used to label phosphate groups, such as at the 5′-end of oligonucleotides, via a carbodiimide reaction using EDC. The resultant phosphoramidate linkage is a common way to modify oligonucleotides at the 5′-end.

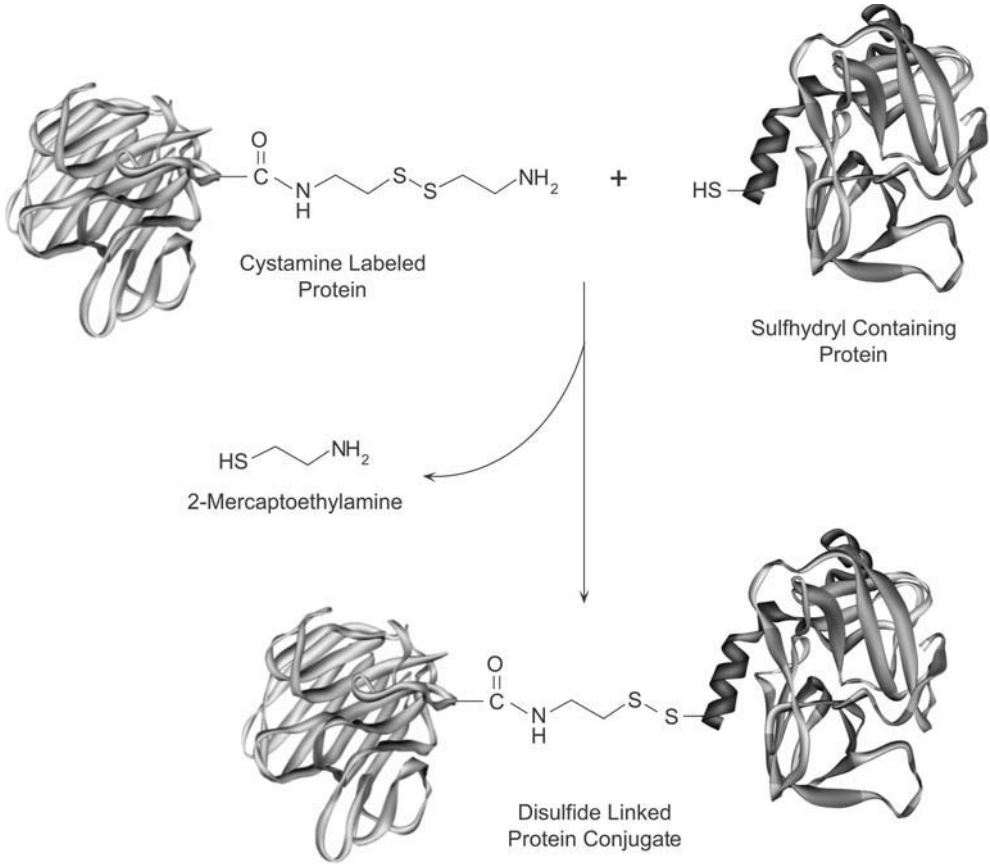


Figure 1.73 The disulfide group of a cystamine-modified protein may undergo disulfide interchange reactions with another sulfhydryl-containing protein to yield a disulfide-linked conjugate.

Using this approach, EGF has been successfully conjugated by disulfide exchange to the A chain of diphtheria toxin (Shimisu *et al*., 1980). A cystaminyl derivative of insulin also could be conjugated to the A chain of diphtheria toxin by this method (Miskimins and Shimizu, 1979). Other references to disulfide exchange using cystamine include Oeltmann and Forbes (1981) and Bacha *et al*. (1983) who prepared antibody–toxin and peptide–toxin conjugates, respectively.

Finally, cystamine may be used to conjugate two macromolecules through its terminal amine groups. In this case, the internal disulfide bridge remains intact, forming a reversible conjugate of the two molecules through reduction of the disulfide bond. Using this approach, the first molecule is modified with cystamine by use of the EDC reaction. A second molecule then is reacted with the free amines of cystamine on the first molecule by use of an amine-reactive chemistry. Typically, this reaction scheme is used if the first molecule initially contains no reactive amines and the second molecule is often an amine-reactive fluorescent tag or other probe. For instance, DNA probes may be cystamine-modified through their 5′-phosphate group using this method and amine-reactive biotin labels subsequently attached. The biotin label is then

reversible by virtue of the cystamine cross-bridge through simple disulfide reduction (Chapter 27, Section 2.2).

Modification of Proteins with Cystamine

The following protocol is useful for the modification of proteins with cystamine with subsequent reduction to create the free sulfhydryl.

Protocol

1. Dissolve the protein to be modified at a concentration of 10 mg/ml in a buffer having a pH between 4.7 and 7.5. Avoid buffers or other components containing competing groups to the carbodiimide reaction (i.e., carboxylates or amines). For the lower pH conditions, 0.1 M MES, pH 4.7 works best. For a physiological pH environment, 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 also will give good incorporation of cystamine. For other concentrations of protein in solution, proportionally adjust the amount of reagents added.
2. Dissolve cystamine (Aldrich) in the reaction buffer at a concentration of 2.25 mg/ml (10 mM). Add an aliquot of this solution to the protein solution to be modified. Use about a 10- to 20-fold molar excess of cystamine over the amount of protein present. For a protein of MW 100,000 at a concentration of 10 mg/ml, add 10 µl of the stock cystamine solution to each ml of protein solution to obtain a 10-fold molar excess.
3. Add EDC (Thermo Fisher) to the solution prepared in (2) to obtain at least a 5-fold molar excess over the amount of cystamine present. React for 2 hours at room temperature.
4. Separate excess cystamine and EDC (and reaction by-products) from the modified protein by dialysis or gel filtration using 10 mM sodium phosphate, 0.15 M NaCl, pH 7.2. A desalting column may be used for the gel filtration procedure (i.e., Zeba spin columns from Thermo Fisher).
5. To reduce the disulfide groups, add DTT at a concentration of 0.5 mg DTT per mg of modified protein. A stock solution of DTT may be prepared to make it easier to add it to a small amount of protein solution. In this case, dissolve 20 mg of DTT per ml of 0.1 M sodium acetate, 0.1 M NaCl, pH 4.5. Add 25 µl of this solution per mg of modified protein.
6. Mix and react at room temperature for 30 minutes.
7. Purify the thiolated protein from excess DTT by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.2. The modified protein should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide crosslinks.

Modification of Nucleic Acids and Oligonucleotides with Cystamine

DNA or RNA also may be modified with cystamine at the 5′-phosphate group using a carbodiimide reaction. See Chapter 27, Section 2.2 for a complete discussion of the labeling protocol.

Use of Disulfide Reductants

One of the most convenient ways of generating sulfhydryl groups is by reduction of indigenous disulfides. Many proteins contain cystine disulfides that are not critical to structure or activity.

In some cases, mild reducing conditions can free one or more —SH groups for conjugation or modification purposes. The creation of free sulfhydryls in this manner allows for site-directed modification at a limited number of locations within the protein molecule.

This method of creating sulfhydryls for conjugation purposes should be avoided, however, if the indigenous disulfides are important for maintaining native structure and activity. Disulfides are often the point of attachment for subunits within a protein molecule. The cystine bonds may be crucial for maintaining quaternary integrity. Reduction may cause a protein to break up into two or more subunits with little or no remaining activity. Disulfides also may be critical for retention of ligand binding activity. Deformation of an active site may occur if important disulfides are reduced. In these cases, the best mode of thiolation is through the use of a reagent system that does not require a disulfide reducing agent, such as 2-iminothiolane or SATA (see previous discussion, this section).

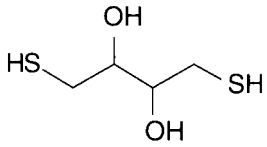
Occasionally, even a protein containing critical disulfides can be partially reduced to yield a useful thiolated derivative. IgG molecules contain disulfide groups that hold together the two heavy chains as well as disulfides holding the light chain–heavy chain pairs together. Selective reduction of some or all of the hinge region disulfides between the heavy chains can result in a divalent or even a monovalent antibody molecule that still maintains its antigen binding capability. Reductants such as DTT, 2-mercaptoethylamine, 2-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP) in a non-denaturing environment can be used at low concentrations to perform this type of partial cleavage. The thiolated "half" antibody so generated then can be successfully conjugated with enzymes or other molecules through the sulfhydryl residue(s) in the exposed hinge region (Chapter 20, Section 1.1).

Disulfide reductants also are used to investigate protein structural properties. In this case, retention of activity is not the critical issue, but complete reduction of all disulfides is paramount. The standard method of doing protein subunit molecular weight determinations by SDS polyacrylamide gel electrophoresis often depends on complete disulfide reduction. When total reduction needs to be assured, the reductants must also contain a deforming agent to unfold protein tertiary structure. This is typically done by including high concentrations of denaturants such as 8 M urea or guanidine or detergents such as SDS. Under severely deforming conditions, proteins unfold exposing internal disulfides to the reducing agent. Without these added reagents to deform native protein structure, many buried disulfides would remain unaffected by the reductants.

The following reducing agents represent the most popular options for cleaving disulfide bonds. Their properties and use vary widely. The decision as to which reagent is best often is governed by the molecule being reduced and the potential application. Careful review of these properties may sway the success or failure of a conjugation protocol.

Cleland's Reagent: DTT and DTE

Dithiothreitol (DTT) and dithioerythritol (DTE) are the *trans* and *cis* isomers of the compound 2,3-dihydroxy-1,4-dithiolbutane. The reducing potential of these versatile reagents was first described by Cleland in 1964. Due to their low redox potential (−0.33 V) they are able to reduce virtually all accessible biological disulfides and maintain free thiols in solution despite the presence of oxygen. The compounds are fully water-soluble with very little of the offensive odor of the 2-mercaptoethanol they were meant to replace. Since Cleland's original report, literally thousands of references have cited the use of mainly DTT for the reduction of cystine and other forms of disulfides.



DTT;
Dithiothreitol
MW 154.25

The unique characteristics of DTT and DTE are mainly reflected in their ability to form intramolecular ring structures upon oxidation. Disulfide reductants such as 2-mercaptoethanol, 2-mercaptoethylamine, glutathione, thioglycolate, and 2,3-dimercaptopropanol cleave disulfide bonds in a two-step reaction that involves the formation of a mixed disulfide (Figure 1.74). In the second stage of the reducing process, the mixed disulfide is cleaved by another molecule of reductant, freeing the sulfhydryl and forming a dimer of the reducing agent through the formation of an intermolecular disulfide bond. For simple reductants containing only one thiol, the equilibrium for disulfide exchange is nearly equivalent for the reductant and target protein. Thus, monothiol compounds are usually required in extreme excess to drive the reaction to completion.

The presence of two sulfhydryl groups in DTT and DTE, however, allows the formation of a favored cyclic disulfide during the course of target protein reduction (Figure 1.75). This drives the equilibrium toward the reduction of target disulfides. Therefore, complete reduction is possible with much lower concentrations of DTT or DTE than when using monothiol systems.

As with all reductants, DTT and DTE will reduce disulfides only if they are accessible. The three-dimensional structure of a protein molecule often contains disulfides buried deep in the inner structure of the polypeptide chains. A protein retaining its native conformation is frequently protected from complete reduction. In the absence of denaturants such as urea, guanidine or SDS, DTT is not capable of reducing all available disulfides within some proteins

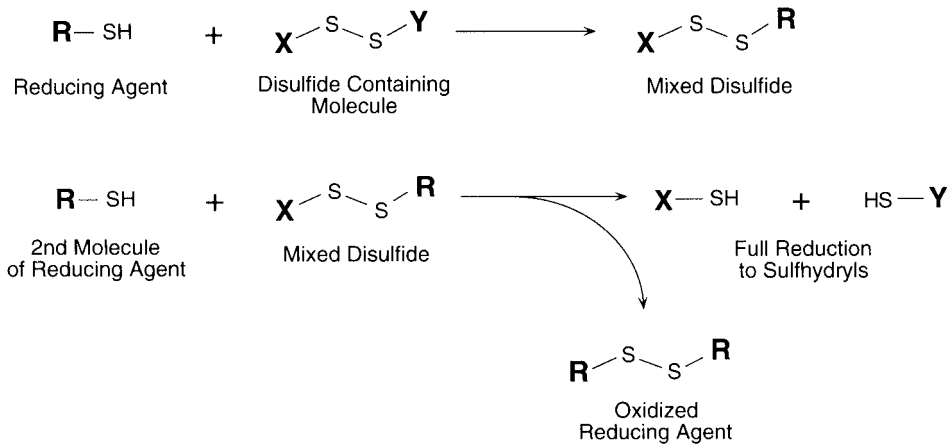


Figure 1.74 Thiol-containing disulfide reductants reduce disulfide groups through a multi-step process producing a mixed disulfide intermediate.

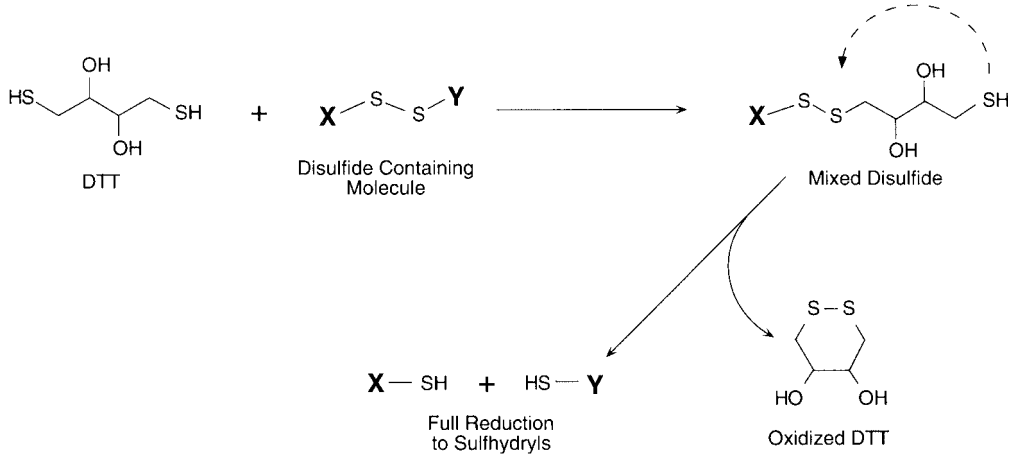


Figure 1.75 DTT is highly efficient at reducing disulfides, since a single molecule can reduce the intermediate mixed disulfide by forming a ring structure.

(Bewley *et al.*, 1968; Bewley and Li, 1969). For instance, at moderate concentrations of DTT and no denaturants, limited cleavage of disulfides in antibody molecules can result in reducing mainly the bonds between the heavy chains of the immunoglobulin. This produces two half-antibody molecules, each containing one antigen binding site and free sulfhydryls in the hinge region. This limited reduction process can be used to site-direct sulfhydryl-reactive conjugation reagents away from the antigen binding sites, thus preserving activity (de Rosario *et al.*, 1990). However, using an appropriate concentration of deforming agents, DTT efficiently reduces all protein disulfides in the antibody and allows subunit separation for analysis (Konigsberg, 1972).

In a comparative study of disulfide reducing agents, it was determined that use of the relatively strong reductants DTT and TCEP required only 3.25 and 2.75 mole equivalents per mole equivalent of antibody molecule to achieve the reduction of two interchain disulfide bonds between the heavy chains of a monoclonal IgG (Sun *et al.*, 2005). This limited reduction strategy retains intact bispecific antibody molecules while providing discrete sites for conjugation to thiols.

DTT also may be used to cleave disulfide containing modification and crosslinking reagents. For thiolation procedures, DTT may be used to remove a dithiopyridyl group or cleave other disulfides to produce a free sulfhydryl. In this case, the presence of a denaturant usually is not required to access and reduce the disulfide of the modification reagent. Similarly, disulfides of crosslinking agents may be reduced after two macromolecules have been conjugated to release them as desired. This technique is often used to analyze receptor–ligand interactions or to discover how two proteins associate *in vivo*.

Complete Reduction of Disulfides in Protein Molecules Using DTT

Protocol

1. Dissolve a disulfide-containing protein or peptide at a concentration of 1–10 mg/ml in 6 M guanidine hydrochloride, 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4. Alternative

denaturant conditions may be used (i.e., 8 M urea or 2.3 percent (w/w) SDS) along with any other buffer salts and pH values desired. A pH between 7.0 and 8.1 usually works best.
2. Add DTT to a final concentration of 10–100 mM.
3. Incubate for 2 hours at room temperature. For some buried disulfides to become exposed and fully reduced, it may be necessary to heat the solution (in a capped test tube) at 50°C for 30 minutes. Some procedures use a 2-minute incubation in a boiling water bath to completely denature the protein.
4. For removal of excess DTT, a protein of molecular weight greater than 5,000 may be isolated by gel filtration using a desalting column. To maintain the stability of the exposed sulfhydryl groups, include 1–10 mM EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

Use of DTT to Cleave Disulfide-Containing Crosslinking Agents

The following method may be used to reduce the disulfide bonds of some crosslinking agents, thus cleaving conjugated proteins. This procedure will reduce the pyridyl disulfide group of SPDP to create a thiolated species (see previous discussion in this section and Chapter 5, Section 1.1). It also may be used to partially reduce the indigenous disulfides in some protein molecules. In this regard, low concentrations of DTT under non-denaturing conditions have been used to selectively reduce the disulfides between the heavy chains of immunoglobulin G (Edelman *et al*., 1968; Sun *et al*., 2005). Without an added denaturant to open up the polypeptide chain, internally buried disulfides typically will remain unreduced.

Protocol

1. Dissolve a crosslinked protein or peptide that has been conjugated with the use of a disulfide-containing crosslinker at a concentration of 1–10 mg/ml in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4. Alternative buffer conditions and pH values may be used, however a pH between 7.0 and 8.1 usually works best.
2. Add DTT to a final concentration of 1–10 mM.
3. Incubate for 2 hours at room temperature.
4. For removal of excess DTT, a protein of molecular weight greater than 5,000 may be isolated by gel filtration using a desalting resin. To maintain the stability of the exposed sulfhydryl groups, include 10 mM EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

2-Mercaptoethanol

2-Mercaptoethanol is one of the most common agents used for disulfide reduction. Sometimes referred to as β-mercaptoethanol, it is a clear, colorless liquid with an extremely strong odor. All operations with this chemical should be performed in a well-ventilated fume hood. The reduction of protein disulfides with 2-mercaptoethanol proceeds rapidly via a two-step process involving an intermediate mixed disulfide (Figure 1.76). Due to its strong reducing properties, the reagent is used most often when complete disulfide reduction is required. It also

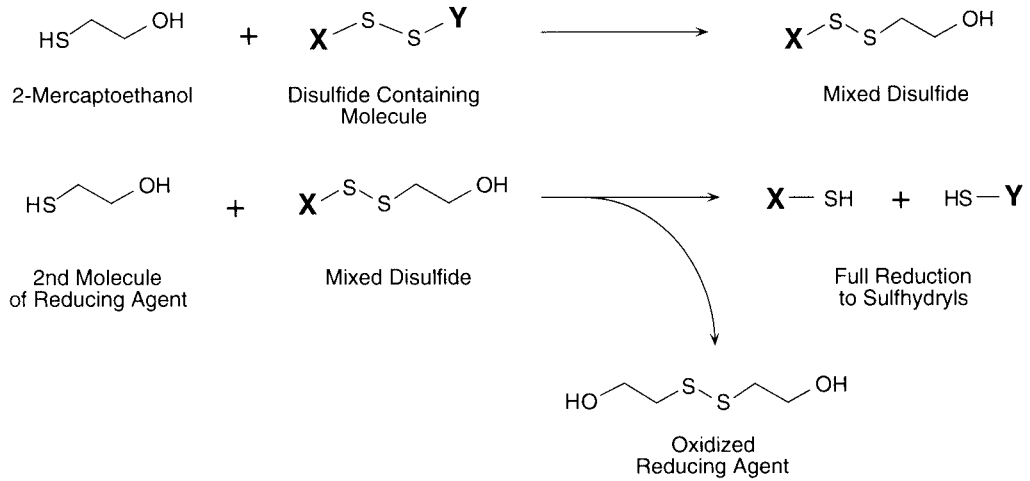
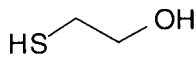


Figure 1.76 The reduction of disulfides by 2-mercaptoethanol proceeds through a mixed disulfide intermediate.

can be used to cleave disulfide-containing crosslinking agents. Usually a concentration of 0.1 M 2-mercaptoethanol will cleave a disulfide-containing crosslinker and liberate conjugated proteins (Chapter 2, Section 2.6)



2-ME;
2-Mercaptoethanol
MW 78.13

2-Mercaptoethanol is used as a reducing additive in a number of biochemical reagents. It is used as a reductant for a Gram-negative bacteria lysis buffer (Schwinghamer, 1980; Scopes, 1982), as the second-dimensional equilibration buffer for 2-D electrophoresis (Dunbar, 1987), as the sample reducing buffer for SDS polyacrylamide gel electrophoresis (Laemmli, 1970), and as a participant in the *o*-phthalaldehyde (OPA) reaction for the detection of primary amines (Jones and Gilligan, 1983).

Protocol for Preparation and Use of a Gram-Negative Bacteria Lysis Buffer

1. Prepare a solution consisting of 2.5 ml glycerol, 100 µl of 10 percent Triton X-100 (Thermo Fisher Surfact-Amps X-100), and 10 µl 2-mercaptoethanol.
2. Add 10 g of wet packed cells to the lysis buffer and stir vigorously for 30 minutes.
3. Add 30 ml of an extraction buffer consisting of 20 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.2 mg/ml lysozyme, and 10 µg/ml DNase I.
4. Add 5 mg PMSF dissolved in 0.5 ml acetone and 0.1 mg pepstatin A.
5. Centrifuge for 20 minutes at 15,000 g. Recover the extracted, solubilized material in the supernatant.

Protocol for Preparation and Use of the Second-Dimension Equilibration Buffer for 2-D Gels

The following procedure relates to electrophoretic protocols where the first dimension is developed by isoelectric focusing (in tube gels) and the second dimension is a size exclusion separation by SDS polyacrylamide electrophoresis in a slab gel.

1. Add 4.0 g SDS and 20 ml of 10 percent glycerol to 150 ml of 0.125 M Tris, pH 6.8, and adjust the final volume to 200 ml. Once dissolved, add a few crystals of bromophenol blue, mix, and pass the solution through a 0.2 µm filter. For storage, freeze in 10–15 ml aliquots.
2. Immediately before use, add 2-mercaptoethanol to a final concentration of 0.5–0.8 percent.
3. Incubate the first-dimensional electrophoresis tube gel in this reducing buffer for 15 minutes. Drain off excess buffer and electrophorese in the second dimension.

SDS Sample Buffer for Running Electrophoresis Size Separations Under Reducing Conditions

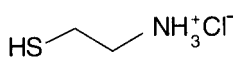
1. Dissolve 2.0 g of SDS, 0.75 g Tris base, and 10 ml of glycerol in 90 ml of water. Adjust the pH to 6.8 and bring the final volume to 100 ml.
2. To a small aliquot of the above buffer, add 2-mercaptoethanol to obtain a final concentration of 2–5 percent. Only 200 µl of this buffer typically is required to treat and reduce about 10–500 µg of protein. Solubilize the protein sample in this buffer.
3. Incubate in a sealed tube at 95°C for 5–10 minutes or in a boiling water bath for 1–2 minutes. Electrophorese immediately.

OPA Solution for the Fluorescent Detection of Primary Amines (see Section 4.3, OPA, this chapter)

1. Add 3 ml of the detergent Brij-35 (as a 30 percent solution) and 2 ml of 2-mercaptoethanol to 950 ml of Fluoraldehyde Reagent Diluent (all reagents from Thermo Fisher).
2. Dissolve 0.5–0.8 g of OPA crystals in about 10 ml of methanol.
3. Mix the OPA solution with the solution from (1) and store under nitrogen in sealed glass bottles at 4°C. The addition of an aliquot of this solution to a sample containing primary amines will yield an intense blue fluorescence.

2-Mercaptoethylamine

2-Mercaptoethylamine (also called aminoethanethiol) is a disulfide reducing agent that has found widespread application in the partial reduction of immunoglobulin molecules. The reagent is supplied as a solid in the hydrochloride form (Thermo Fisher) and possesses very little of the sulfhydryl odor of 2-mercaptoethanol. When used under non-denaturing conditions, 2-mercaptoethylamine can cleave the disulfide bonds between the heavy chains of IgG. This directed reduction is important for generating sulfhydryls while preserving antigen binding activity.



2-MEA;
2-Mercaptoethylamine
Hydrochloride
MW 113.62

The complex structure of an antibody molecule creates two antigen binding sites from the interaction of the hypervariable regions on both the heavy and light chains. For this reason, heavy–light chain pairing must remain intact during any modification procedure to ensure that antigen binding activity is retained. In addition, it is important that any chemistry take place away from the antigen binding sites so they are not sterically blocked by modification reagents or by subsequent conjugation steps. 2-Mercaptoethylamine can be used to cleave disulfides primarily in the hinge region of IgG—away from the antigen binding sites—thus preserving the disulfides that hold the heavy and light chains together (Yoshitake *et al.*, 1979). It also can be used to reduce F(ab′)2 fragments, because they still retain the hinge region disulfides of intact IgG (Figure 1.77).

Once reduced with 2-mercaptoethylamine, immunoglobulins often will be cleaved in half, forming two heavy chain–light chain molecules of MW 75,000–80,000 and each containing one antigen binding site. These half molecules of IgG will possess reactive sulfhydryls in the hinge region that can be used in conjugation protocols with sulfhydryl-reactive crosslinking reagents. For instance, a reduced antibody may be used to make a conjugate with a maleimide-activated enzyme, forming a reagent useful in immunoassays (Chapter 20, Section 1.1). Similarly, F(ab′)2 fragments may be reduced to yield two molecules, each containing an antigen binding site. Making conjugates with this low-molecular-weight fragment can dramatically reduce background in assay systems or provide access to antigens restricted to higher-molecular-weight conjugates made with intact antibody (such as in immuno-histochemical staining techniques).

The use of a 500-fold molar excess of 2-mercaptoethylamine over the concentration of antibody presence was found to result in a partially reduced antibody in which two disulfides were reduced to yield four thiols (Sun *et al.*, 2005). This strategy can be used to retain a biospecific antibody construct for subsequent discrete conjugation at the hinge region between the heavy chains.

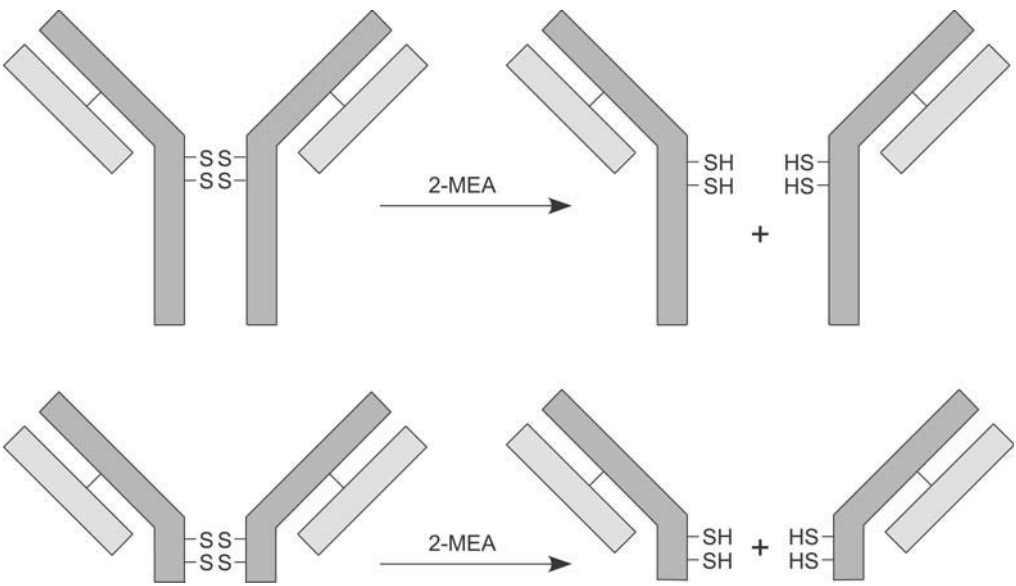


Figure 1.77 Disulfide reducing agents such as 2-mercaptoethylamine can be used to cleave the disulfide bonds in the hinge region of antibody molecules. Either intact IgG molecules or $F(ab')_2$ fragments may be reduced in this manner to yield monofunctional antigen binding fragments.

Protocol

1. Dissolve the antibody to be reduced at a concentration of 10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4, containing 1–10 mM EDTA.
2. To each ml of the antibody solution, add 6 mg of 2-mercaptoethylamine hydrochloride (final concentration is 50 mM). Mix to dissolve. Alternatively, to limit the degree of disulfide reduction, add a 500-fold molar excess of 2-mercaptoethylamine over the concentration of antibody present.
3. Incubate the solution in a sealed tube for 90 minutes at 37°C.
4. Purify the reduced IgG from excess 2-mercaptoethylamine and reaction by-products by dialysis or gel filtration using a desalting resin. All buffers should contain 1–10 mM EDTA to preserve the free sulfhydryls from metal-catalyzed oxidation. The sulfhydryl-containing half antibody now may be used in conjugation protocols that use —SH-reactive heterobifunctional crosslinkers (Chapter 5, Section 1).

TCEP

The reduction of disulfide bonds with trivalent phosphines has been known for sometime (Levison *et al.*, 1969; Ruegg and Rudingder, 1977; Kirley, 1989). Unfortunately, trialkylphosphines generally are water-insoluble, undergo autoxidation, and are extremely odious.

To overcome these issues, the water-soluble TCEP was synthesized and successfully used to cleave organic disulfides to sulfhydryls in water (Burns *et al.*, 1991). The advantage of using this phosphine derivative in disulfide reduction as opposed to previous ones is its excellent stability in aqueous solution, its lack of reactivity with other common functionalities in biomolecules, and its freedom from odor.

The reaction of TCEP with biological disulfides proceeds with initial cleavage of the S—S bond followed by oxidation of the phosphine (Figure 1.78). The stability of the phosphine oxide bond that is formed in this process is great enough to prevent reversal of the reaction. Since this reaction is performed without any added —SH compounds, subsequent conjugation with the generated sulfhydryl groups can be done without removal of excess TCEP or reaction by-products (provided the conjugation step does not involve disulfide exchange reactions, such as with the active disulfide containing reagent SPDP; Chapter 5, Section 1.1).

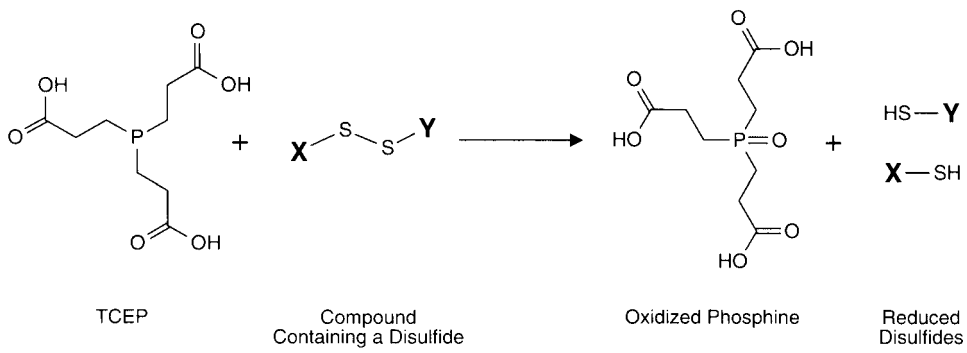
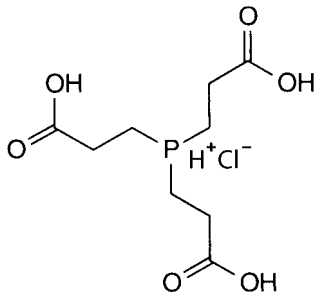


Figure 1.78 TCEP reduction of disulfides proceeds without the use of thiol compounds.

Although TCEP is capable of rapidly and quantitatively reducing simple organic disulfides in solution, it requires the presence of a deforming agent to fully reduce all disulfides in proteins. Without opening up the internal disulfides in many protein molecules, TCEP will not be able to reduce them. For complete reduction of IgG, it was found that 20 mM TCEP and 5 minutes of boiling was needed (Hines, 1992). Partial reduction, however, is possible for some more accessible disulfides in protein using aqueous buffers at room temperature. For instance, the use of a 2.75-fold molar excess of TCEP over the concentration of a monoclonal IgG resulted in the reduction of only two disulfide bonds in the hinge region of the antibody, leaving all other disulfides intact (Sun *et al.*, 2005).



TCEP
Tris(2-carboxyethyl)phosphine
(hydrochloride)
MW 250.19

Protocol for the Complete Reduction of Disulfide Bonds within Protein Molecules

1. Dissolve the protein to be reduced at a concentration of 1–10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. Other buffers and pH values also may be used. A strong denaturant may be added (6 M guanidine or 8 M urea) to this solution to promote protein unfolding and make buried disulfides more accessible.
2. Add TCEP to a final concentration of 20 mM.
3. Place in a sealed tube and incubate in a boiling water bath for 5 minutes. If a denaturant was included in the buffer from (1), then high temperature may not be necessary. Alternatively, incubate the sample at 50°C for 30 minutes.
4. To remove excess TCEP and reaction by-products, dialyze the solution or purify by gel filtration using a buffer containing 1–10 mM EDTA.

Protocol for Partial Reduction of Protein Disulfides or for Cleaving Disulfide Containing Modification Reagents

1. Dissolve the protein to be reduced at a concentration of 1–10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4. Other buffers and pH values also may be used. Do not add a denaturant to unfold protein structure.

2. Add TCEP to a final concentration of 20 mM. For partial reduction of antibody disulfides in the hinge region while maintaining a biospecific IgG molecule, add TCEP in a 2.75-fold molar excess over that of the antibody concentration.
3. Incubate for 2 hours at room temperature or 37°C.
4. To remove excess TCEP and reaction by-products, dialyze the solution or purify the protein by gel filtration using a buffer containing 1–10 mM EDTA.

Immobilized Disulfide Reductants

Many extracellular proteins like immunoglobulins, protein hormones, serum albumin, pepsin, trypsin, ribonuclease, and others contain one or more indigenous disulfide bonds. For functional and structural studies of proteins, it is often necessary to cleave these disulfide bridges. Disulfide bonds in proteins are commonly reduced with small, soluble mercaptans, such as DTT, TCEP, 2-mercaptoethanol, thioglycolic acid, cysteine, etc. High concentrations of mercaptans (molar excess of 20- to 1,000-fold) are usually required to drive the reduction to completion.

Cleland (1964) showed that DTT and DTE are superior reagents in reducing disulfide bonds in proteins (see previous discussion, this section). DTT and DTE have low oxidation–reduction potential and are capable of reducing protein disulfides at concentrations far below that required with 2-mercaptoethanol. However, even these reagents have to be used in approximately 20-fold molar excess in order to get close to 100 percent reduction of a protein.

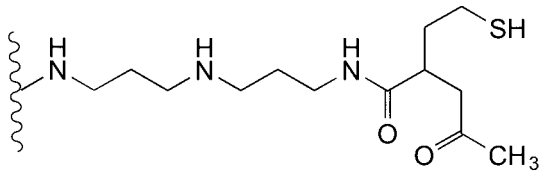
An immobilized disulfide reductant usually consists of an insoluble beaded support material such as agarose that has been modified with a small ligand containing a terminal sulfhydryl group. The presence of densely coupled sulfhydryl groups on the matrix creates enormous disulfide reducing potential. Simply mixing a solution of a disulfide-containing peptide or protein with the immobilized reductant efficiently breaks any disulfide linkages and creates free sulfhydryls. This is done without extraneous sulfhydryl contamination by the reductant, as in the case of soluble reductants.

The use of immobilized disulfide reductants thus has the following advantages over solution phase agents:

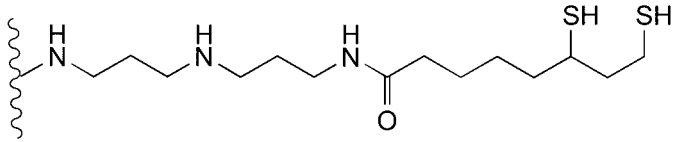
1. Immobilized disulfide reductants can be used to reduce all types of biological disulfides without liberating product or by-product contaminants.
2. Soluble components that interfere with the assay of free thiol groups are not present if immobilized disulfide reductants are used.
3. Small molecules containing disulfide bonds (such as cystine-containing peptides) may be reduced and isolated simply by removing the immobilized reductant. Separation of reduced molecules from reductant is much more difficult if a soluble reducing agent is used with low-molecular-weight disulfides.
4. Immobilized disulfide reductants easily can be regenerated and reused many times.

Immobilized dihydrolipoamide (thioctic acid) (Gorecki and Patchornick, 1973; Gorecki and Patchornick, 1975) and immobilized *N*-acetyl-homocysteine thiolactone (Eldjarn and Jellum, 1963; Jellum, 1964) are the two most commonly used immobilized disulfide reductants. In addition, immobilized TCEP provides a reducing matrix that is free of thiols (Thermo Fisher). Such immobilized reductants successfully can be used to reduce many types of biological disulfides, including small molecules like oxidized glutathione and bovine insulin. They

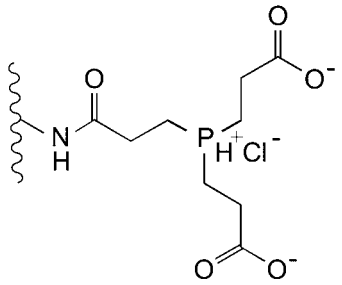
are particularly convenient to reduce peptide disulfides prior to conjugation, which may be necessary even with peptides labeled at their end with a cysteine group, as the sulfhydryl may become oxidized over time and form an inter-peptide disulfide bridge.



Immobilized N-acetyl homocysteine
attached to a diaminodipropylamine spacer



Immobilized dihydrolipoamide attached
to a diaminodipropylamine spacer



Immobilized TCEP attached
via an amide bond

Immobilized disulfide reductants may be synthesized as described in Hermanson *et al.* (1992) or obtained commercially (Thermo Fisher).

A. Reduction of Peptides Using Immobilized Reductants

Note: For optimal reduction of peptides, the following steps should be performed at room temperature.

1. Pack an immobilized reductant gel (2 ml settled gel) in a disposable polypropylene column and wash with 5 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA (equilibration buffer).

2. Prepare the sulfhydryl column by washing with a disulfide reducing agent. Apply 10 ml of freshly made 10 mM DTT solution (15.4 mg of DTT dissolved in 10 ml of equilibration buffer). This treatment converts the immobilized ligands into a fully reduced form (free —SH groups).
3. Wash the column with 20 ml of equilibration buffer-1 to remove free DTT.
4. Apply to the column 1.0 ml of peptide solution (dissolved in equilibration buffer) to be reduced. Normally, small peptides (molecular weight less than or equal to that of insulin) require no deforming agent (denaturant) such as guanidine to be completely reduced.
5. After the sample has completely entered into the gel bed, wash the column with 9 ml of equilibration buffer, while collecting 1.0 ml fractions.
6. Monitor the elution of reduced peptide from the column by measuring the absorbance at 280 nm (if peptide absorbs at this wavelength) as well as by performing an Ellman's assay (Section 4.1, this chapter) for sulfhydryl groups using a small aliquot (10–20 µl) of each collected fraction.
7. Regenerate the sulfhydryl containing support by following steps 2 and 3 above. Such columns can be regenerated and reused at least 10 times without any significant decrease in the reductive capacity.
8. Store the column in 0.02 percent sodium azide at 4°C.

B. Reduction of Proteins Using Immobilized Reductants

Note: For optimal reduction of proteins, the following steps must be performed at room temperature.

1. Pack an immobilized reductant gel (2 ml) in a disposable polypropylene column and wash with 5 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA (equilibration buffer-1).
2. Prepare the sulfhydryl column by washing with a disulfide reducing agent. Apply 10 ml of freshly made 10 mM DTT solution (15.4 mg of DTT dissolved in 10 ml of equilibration buffer-1).
3. Wash the column with 10 ml of equilibration buffer-1 and 10 ml of 0.1 M sodium phosphate buffer, pH 8.0 containing 1 mM EDTA and 6 M guanidine hydrochloride (equilibration buffer-2) to remove free DTT.
4. Apply to the column 1.0 ml of protein solution (dissolved in equilibration buffer-2) to be reduced. The inclusion of a denaturant in the solution deforms the protein structure so that inner disulfides are available to the immobilized reductant. Without the presence of guanidine or another deforming agent (i.e., urea, SDS, etc.), only partial reduction of the protein is possible.
5. After the sample has completely entered the gel bed, incubate the column at room temperature for 1 hour.
6. Wash the column with 9 ml of equilibration buffer-2 while 2 ml fractions are collected.
7. Monitor elution of reduced protein from the column by measuring the absorbance at 280 nm as well as by performing an Ellman's assay for sulfhydryl groups (Section 4.1, this chapter) using a small aliquot (50–100 µl) of each collected fraction.
8. Regenerate the sulfhydrylcontaining column by following steps 2 and 3 above. Such columns can be regenerated and reused at least 10 times without any significant decrease in the reductive capacity.
9. Store the column in 0.02 percent sodium azide at 4°C.

Sodium Borohydride

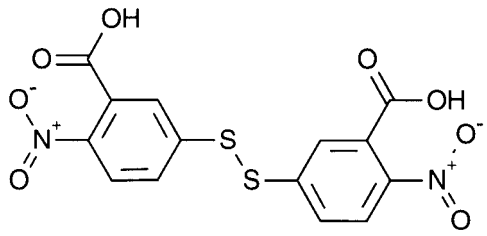
Perhaps the simplest route to the reduction of disulfide groups in peptides is the use of sodium borohydride ($NaBH_4$). This common reducing agent often used in organic synthesis is able to specifically reduce disulfides to free thiols without affecting any of the other major functional groups in proteins. Gailit (1993) developed a protocol for borohydride reduction, which avoids any purification steps to remove the reducing agent after the reaction. Thus, peptides reduced by this protocol can be used immediately in bioconjugate applications without additional steps.

Protocol

1. Dissolve the peptide to be reduced in a buffer at pH 8–10. Sodium phosphate or sodium bicarbonate at 0.1 M work well. The optimal pH range for borohydride activity is alkaline, therefore avoid using buffers at neutral pH.
2. Add sodium borohydride (Aldrich) to the peptide solution to obtain a final concentration of 0.1 M. Generation of hydrogen bubbles will occur as the borohydride is dissolved.
3. Incubate at room temperature for 30–60 minutes.
4. Adjust the pH of the reaction to pH 4.0 using dilute HCl. Incubate for 10 minutes to assure the complete destruction of excess borohydride. Hydrogen bubbles again will be evolved from the solution.
5. Readjust the pH to the optimal value for the bioconjugate application to be done using the generated thiols. Use the reduced peptide immediately to prevent reoxidation of the thiols to disulfides.

Ellman's Assay for the Determination of Sulfhydryls

Ellman's reagent, 5,5′-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), reacts with sulfhydryls under slightly alkaline conditions to release the highly chromogenic compound, 5-thio-2-nitrobenzoic acid (TNB) (Ellman, 1959; Riddles *et al.*, 1979) (Figure 1.79). The reagent contains a disulfide bond between two TNB groups, and reacts with free sulfhydryls to create a mixed disulfide product. The target of the reaction is the unprotonated, conjugate base form of the thiol, R—S^-. At pH 8.0, the release of one TNB group per available thiol provides a yellow-colored product with an extinction coefficient at 412 nm of 13,600 $M^{-1}cm^{-1}$. The increase in absorbance at this wavelength is directly proportional to the concentration of sulfhydryls in solution. Correlation to a standard curve of known sulfhydryl concentrations allows accurate measurement of thiol content in unknown samples.



Ellman's Reagent
5,5'-Dithio-bis-(2-nitrobenzoic acid)
MW 396.4

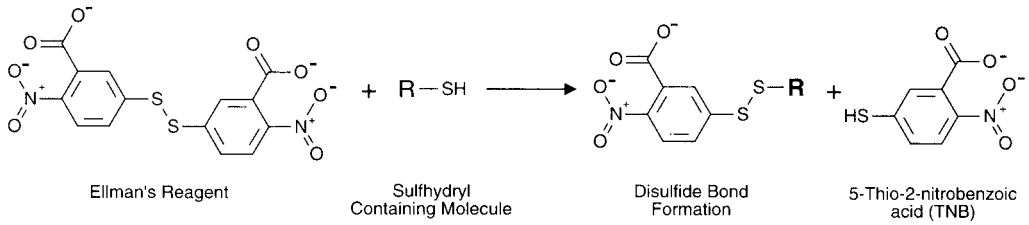


Figure 1.79 The reaction of Ellman's reagent with a sulfhydryl group releases the chromogenic TNB anion, which can be quantified by its absorbance at 412 nm.

Ellman's reagent has been used not only for the determination of sulfhydryls in proteins and other molecules, but also as a pre-column derivatization reagent for the separation of thiol compounds by HPLC (Kuwata *et al.*, 1982), in the study of thiol-dependent enzymes (Tsukamoto and Wakil, 1988; Alvear *et al.*, 1989; Masamune *et al.*, 1989), and to create sulfhydryl-reactive chromatography supports for the coupling of affinity ligands (Jayabaskaran *et al.*, 1987). Another important use of the compound is in the assessment of conjugation procedures using sulfhydryl-reactive crosslinking agents (Chapter 5, Section 1).

Depending on the conditions, an Ellman's assay can detect as little as 10 nM cysteine concentration. The linearity can extend into the mM range, making the test extremely flexible for different sample situations.

Protocol

1. Dissolve Ellman's reagent (Thermo Fisher) in 0.1 M sodium phosphate, pH 8.0, at a concentration of 4 mg/ml.
2. Prepare a set of standards by dissolving cysteine in 0.1 M sodium phosphate, pH 8.0, at an initial concentration of 2 mM (3.5 mg/ml) and serially diluting this solution (1:1) with reaction buffer down to at least 0.125 mM. This will produce five solutions of cysteine for generating a standard curve. If a more dilute concentration range is required, continue to serially dilute until a set of standards in the desired range is obtained.
3. Label a set of tubes according to the standards and samples to be used. Add 250 µl of each standard and sample to the appropriate tubes. If the samples are in a buffer that may significantly change the pH of the reaction buffer, the samples should be buffer-exchanged or dialyzed into 0.1 M sodium phosphate, pH 8.0, before running the assay.
4. Add 50 µl of Ellman's reagent to each standard and sample tube. Mix well.
5. Incubate at room temperature for 15 minutes.
6. Measure the absorbance of each solution at 412 nm.
7. Plot the absorbance versus cysteine concentration for each of the standards. Determine the sulfhydryl concentration of the samples by comparison to the standard curve.

4.2. Introduction of Carboxylate Groups

Modification of various functional groups in macromolecules with the following types of reagents will introduce carboxylate functions for further derivatization purposes. Amines,

sulfhydryls, histidine, and methionine side chains are readily modified to contain short molecules terminating in a carboxylic acid. The short chain can serve as a spacer to enhance steric accommodations and the terminal carboxylate group can facilitate subsequent couplings with amines or hydrazides. The introduction of carboxylates also affects the overall charge characteristics or pI of the molecule being derivatized. The modification of amine residues by acylation with anhydrides not only eliminates the positive charge contribution of the protonated amine, but also adds the negative charge contribution of the acid. The result may be a change of minus two in net charge per group modified. While the reactions involved in such derivatizations are conducted under relatively mild conditions, severe alterations in net charge may cause some macromolecules, like proteins, to denature or lose activity. In addition, if the group being modified happens to be critical for active center operation then functionality may be compromised regardless of conditions. While the following reactions are facile and efficient, it should be kept in mind that in certain instances modification may lead to inactivity.

Modification of Amines with Anhydrides

Acid anhydrides, as their name implies, are formed from the dehydration reaction of two carboxylic acid groups (Figure 1.80). Anhydrides are highly reactive toward nucleophiles and are able to acylate a number of the important functional groups of proteins and other macromolecules. Upon nucleophilic attack, the anhydride yields one carboxylic acid for every acylated product. If the anhydride was formed from monocarboxylic acids, such as acetic anhydride, then the acylation occurs with release of one carboxylate group. However for dicarboxylic acid anhydrides, such as succinic anhydride, upon reaction with a nucleophile the ring structure of the anhydride opens, forming the acylated product modified to contain a newly formed carboxylate group. Thus, anhydride reagents may be used to both block functional groups and to convert an existing functionality into a carboxylic acid.

Protein functional groups able to react with anhydrides include the α-amines at the N-terminals, the ε-amine of lysine side chains, cysteine sulfhydryl groups, the phenolate ion of tyrosine residues, and the imidazolyl ring of histidines. However, acylation of cysteine, tyrosine, and histidine side chains forms unstable complexes that are easily reversible to regenerate the original group. Only amine functionalities of proteins are stable to acylation with anhydride reagents (Fraenkel-Conrat, 1959; Smyth, 1967).

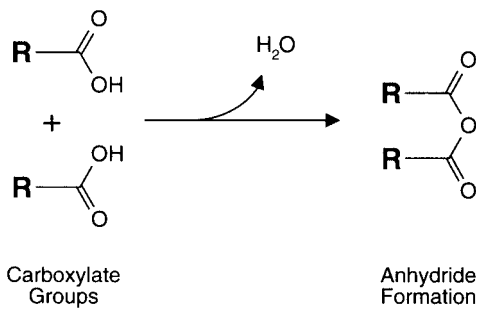


Figure 1.80 Anhydrides are created from two carboxylate groups by the removal of one molecule of water.

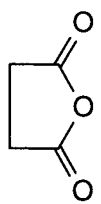
Another potential site of reactivity for anhydrides in protein molecules is modification of any attached carbohydrate chains. In addition to amino group modification in the polypeptide chain, glycoproteins may be modified at their polysaccharide hydroxyl groups to form ester derivatives. Esterification of carbohydrates by acetic anhydride, especially cellulose, is a major industrial application for this compound. In aqueous solutions, however, esterification may be a minor product, since the oxygen of water is about as strong a nucleophile as the hydroxyls of sugar residues.

The major side reaction to the desired acylation product is hydrolysis of the anhydride. In aqueous solutions anhydrides may break down by the addition of one molecule of water to yield two carboxylate groups. The presence of an excess of the anhydride in the reaction medium usually is enough to minimize the effects of competing hydrolysis.

Since both hydrolysis and acylation yield the release of carboxylic acid functionalities, the medium becomes acidic during the course of the reaction. This requires either the presence of a strongly buffered environment to maintain the pH or periodic monitoring and adjustment of the pH with base as the reaction progresses.

Succinic Anhydride

Succinic acid is a four carbon molecule with carboxylic acid groups on both ends. The anhydride has a five-atom cyclic structure that is highly reactive toward nucleophiles, especially amines. Attack of a nucleophile at one of the carbonyl groups opens the anhydride ring, forming a covalent bond with that carbonyl and releasing the other to create a free carboxylic acid (Klotz, 1967). Succinylation of positively charged amino groups of proteins and other molecules thus creates amide bond derivatives and converts the cationic site into a negatively charged carboxylate (Figure 1.81). Succinylated proteins often experience dramatic changes in their three-dimensional structure. Subunits may dissociate (Klotz and Keresztes-Nagy, 1962), enzymatic activity may be compromised (Riordan and Valle, 1963, 1964), and the molecular radius and viscosity may be increased (Habeeb *et al.*, 1958). Other effects on protein conformation and function have been studied as well (Meighen *et al.*, 1971; Shiao *et al.*, 1972; Shetty and Rao, 1978).



Succinic Anhydride
MW 100

Succinic anhydride also may react with protein phenolate side chains of tyrosine residues and the —OH group of aliphatic hydroxy amino acids (Figure 1.82). The phenolate ester derivatives are unstable above pH 5.0, whereas the serine and threonine esters are more stable but may be cleaved by treatment with hydroxylamine at basic pH (Gounaris and Perlman, 1967).

A succinylated casein derivative that has nearly all its amines blocked can be used as a substrate in protease assays (Hatakeyama *et al.*, 1992). As the casein is degraded by a protease, free amines are created from α-chain cleavage and release of α-amino groups. The creation of

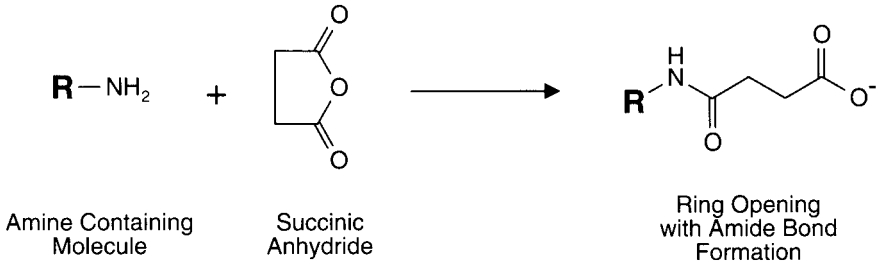


Figure 1.81 Succinic anhydride reacts with primary amine groups in a ring-opening process, creating an amide bond and forming a terminal carboxylate.

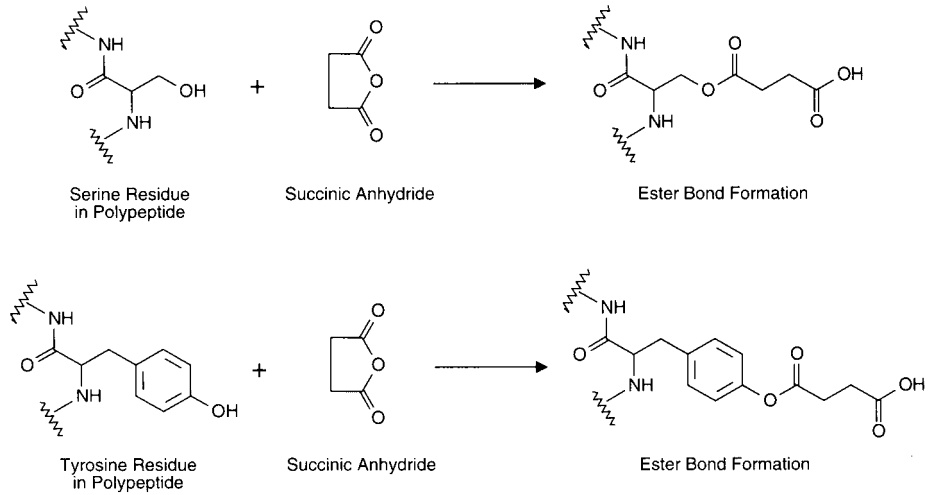


Figure 1.82 The hydroxyl group of serine residues and the phenolate ring of tyrosine groups may be modified with succinic anhydride to produce relatively unstable ester bonds. In aqueous conditions these reactions are minor due to competing hydrolysis by water.

amines can be monitored by an amine detection reagent such as trinitrobenzene sulfonic acid (TNBS; Chapter 1, Section 4.3). The procedure forms the basis for a highly sensitive assay for protease activity.

Succinylated derivatives of nucleic acids may be prepared by reaction of the anhydride with available —OH groups. The reaction forms relatively stable ester derivatives that create carboxylates on the nucleotide for further conjugation or modification (Figure 1.83). This method has been used in nucleic acid synthesis (Matteucci and Caruthers, 1980) and to derivatize nucleotide analogs such as AZT (Tadayoni *et al.*, 1993).

Succinic anhydride also is a convenient extender for creating spacer arms on chromatography supports. Supports derivatized with amine-terminal spacers may be succinylated to totally block the amine functionalities and form terminal carboxylic acid linkers for coupling amine-containing affinity ligands (Cuatrecasas, 1970).

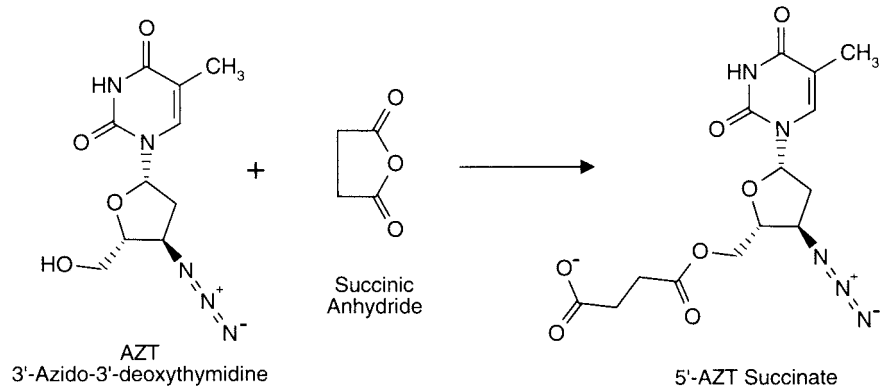


Figure 1.83 Succinic anhydride has been used in nonaqueous conditions to modify the 5′-hydroxyl group of nucleic acid derivatives such as AZT.

Molecules modified with succinic anhydride to create terminal carboxylate functionalities may be further conjugated to amine-containing molecules by use of amide bond forming reagents such as carbodiimides (Chapter 3, Section 1).

Protocol

1. Dissolve (or suspend in the case of insoluble polymers or support materials) the amine-containing molecule to be succinylated in a buffer having a pH between 6.0 and 9.0. Higher pH buffers will cause the reaction to occur faster and result in more amines in an unprotonated state. Suitable buffer salts include sodium acetate, sodium phosphate, and sodium carbonate in a 0.1–1.0 M concentration. Avoid buffers containing primary amine groups such as Tris. Alternatively, the substance may be dissolved in water and the pH maintained in the proper range by periodic addition of NaOH. This is conveniently done by means of a pH stat. Even in buffered reactions, the pH should be monitored to prevent severe acidification of the reaction solution, which could damage the molecule being modified.
2. Add a quantity of succinic anhydride to the reaction medium to provide at least a 5–10 molar excess of reagent over the amount of amines to be modified. Even greater molar excess may be required for total blocking of all the amines of some proteins. When adding solid succinic anhydride, multiple additions may be done to maintain solubility of the reagent in the reaction solution. The anhydride also may be dissolved in dry dioxane before addition to aid in dissolution.
3. React for at least 1–2 hours at room temperature. To assure complete blocking of all amine groups, the reaction may be continued overnight.
4. Remove excess reactants from the succinylated molecule by dialysis, gel filtration, or some other suitable method. The efficiency of amine modification may be assessed by use of the TNBS test for amines (Section 4.3, this chapter). A negative test for amines indicates complete succinylation.

Glutaric Anhydride

Glutaric acid is a linear, five carbon molecule with carboxylic acid groups on both ends. It contains one additional carbon in length than the similar compound succinic acid. The anhydride

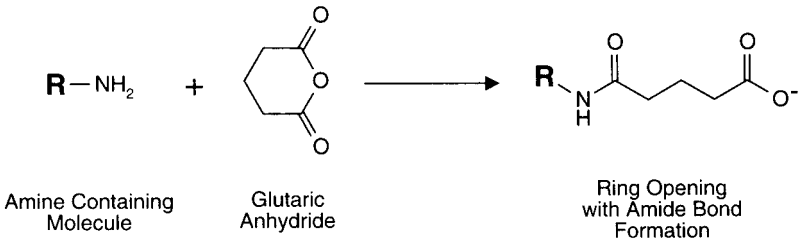
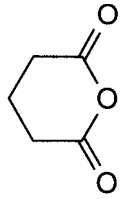


Figure 1.84 Glutaric anhydride reacts with amines in a ring-opening process to create an amide bond linkage and a terminal carboxylate group.

of glutaric acid forms a cyclic structure containing six atoms. Attack of a nucleophile, such as an amino group, on one of the carbonyl groups of glutaric anhydride opens the ring, forming an amide linkage and liberating the other carboxylic acid (Figure 1.84). Reaction with the phenolate of tyrosine or the sulfhydryl group of cysteine forms unstable linkages (an ester and a thioester, respectively) that can easily hydrolyze. As with succinic anhydride, however, aliphatic hydroxyl groups such as those of serine and threonine may be modified with glutaric anhydride to create more stable ester bonds (see above).



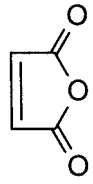
Glutaric Anhydride
MW 114

Protocol

The procedure for the modification of amine-containing compounds with glutaric anhydride is identical to that described for succinic anhydride, above.

Maleic Anhydride

Maleic acid is a linear four carbon molecule with carboxylate groups on either end similar to succinic acid, but with a double bond between the central carbon atoms. The anhydride of maleic



Maleic Anhydride
MW 98

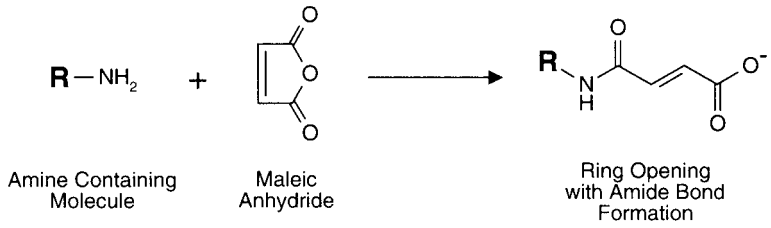


Figure 1.85 Maleic anhydride reacts with amine groups in a ring-opening process to create carboxylate derivatives.

acid is a cyclic molecule containing five atoms in its ring. Although the reactivity of maleic anhydride is similar to other such reagents like succinic anhydride, the products of maleylation are much more unstable toward hydrolysis, and the site of unsaturation lends itself to additional side reactions. Acylation products of amino groups with maleic anhydride are stable at neutral pH and above, but they readily hydrolyze at acid pH values (around pH 3.5) (Butler *et al.*, 1967). Maleylation of sulfhydryls and the phenolate of tyrosine are even more sensitive to hydrolysis.

As with other cyclic anhydrides, the acylation of an amine residue proceeds with elimination of the potential positive charge of the amine and addition of the negative charge created by the anhydride ring opening (Figure 1.85). Thus, a molecule can undergo a change of minus two in net charge per site of maleylation. Proteins extensively modified with maleic anhydride may spontaneously dissociate into subunits or experience a general opening of their three-dimensional structures (Sia and Horecker, 1968; Uyeda, 1969).

The double bond of maleic anhydride may undergo free radical polymerization with the proper initiator. Polymers of maleic anhydride (or copolymers made with another monomer) are commercially available (Polysciences). They consist of a linear hydrocarbon backbone (formed from the polymerization of the vinyl groups) with cyclic anhydrides repeating along the chain. Such polymers are highly reactive toward amine-containing molecules.

Maleic acid imides (maleimides) are derivatives of the reaction of maleic anhydride and ammonia or primary amine compounds. The double bond of a maleimide may undergo an alkylation reaction with a sulfhydryl group to form a stable thioether bond (Chapter 2, Section 2.2). Maleic anhydride may presumably undergo the same reaction with cysteine residues and other sulfhydryl compounds.

Proteins derivatized with maleic anhydride exhibit an increase in their absorptivity at wavelengths below 280 nm, due to the addition of the unsaturated carbon–carbon bond. The extent of maleylation may be estimated by measuring the absorbance increase before and after modification (Freedman *et al.*, 1968).

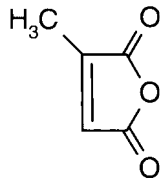
Protocol

Modification of amines with maleic anhydride is done essentially the same as that described for succinic anhydride (this section, Part A), except the pH of the reaction should be kept alkaline (pH 8–9) at all times to prevent unwanted de-acylation. Deblocking of maleylated amines can be accomplished according to the following procedure of Butler *et al.* (1967).

1. Adjust the pH of the maleylated protein or other molecule to pH 3.5 with formic acid and aqueous NH_3.
2. Incubate the solution at 37°C for 30 hours.
3. Stop the deblocking reaction by the addition of NaOH to raise the pH back to neutrality.

Citraconic Anhydride

Citraconic anhydride (or 2-methylmaleic anhydride) is a derivative of maleic anhydride that is even more reversible after acylation than maleylated compounds. At alkaline pH values (pH 7–8) the reagent effectively reacts with amine groups to form amide linkages and a terminal carboxylate. However, at acid pH (3–4), these bonds rapidly hydrolyze to release citraconic acid and free the amine (Figure 1.86) (Dixon and Perham, 1968; Habeeb and Atassi, 1970; Klapper and Klotz, 1972; Shetty and Kinsella, 1980). Thus, citraconic anhydride has been used to temporarily block amine groups while other parts of a molecule are undergoing derivatization. Once the modification is complete, the amines then can be unblocked to create the original structure.



Citraconic
Anhydride
MW 113

Acid labile, heterobifunctional crosslinking reagents have been synthesized using 2-methylmaleic anhydride at one end (Blattler *et al.*, 1985). Amines can be reacted with the anhydride end under alkaline conditions to form amide linkages. The other end, containing another functionality, in this case a maleimide group, is then made to react with a sulfhydryl-containing molecule. After the conjugation is complete, the citraconylamide end can be specifically released by lowering the pH.

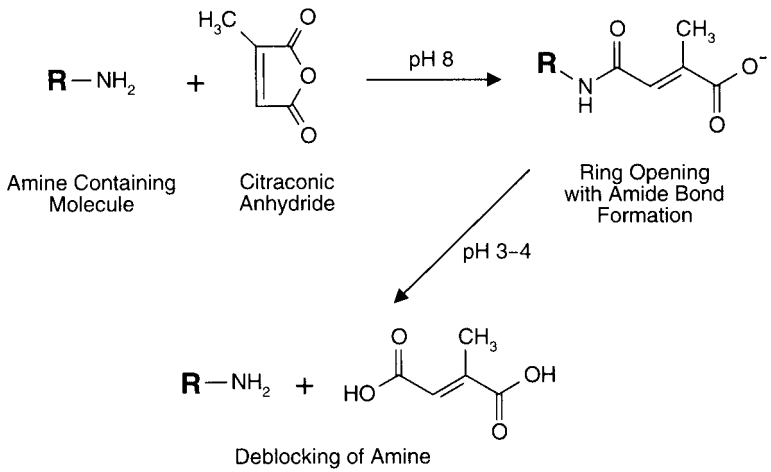


Figure 1.86 Citraconic anhydride can be used to block amine groups reversibly. The amide bond derivative is unstable to acidic conditions.

Citraconic anhydride also has been used to reverse the effects of formalin fixation in tissue sections. Namimatsu *et al.* (2005) found that heating deparafinized tissue sections in a dilute solution of citraconic anhydride broke the formaldehyde crosslinks and restored antigen recognition of proteins within the samples.

Citraconic anhydride is a toxic liquid that should be handled with extreme care in a fume hood. Avoid contact with skin, eyes, or inhalation of vapors.

Protocol

1. Dissolve the amine-containing molecule to be modified in a buffer having a pH between 8 and 9. Maintenance of this pH range is necessary due to the high tendency of citraconylamides to hydrolyze at lower pH. Suitable buffer salts include sodium phosphate and sodium carbonate in a 0.1–1.0 M concentration. Avoid buffers containing primary amine groups such as Tris. Also avoid thiol reducing agents containing —SH groups, as these may be acylated by the anhydride. Alternatively, the substance may be dissolved in water and the pH maintained in the proper range by periodic addition of NaOH. This is conveniently done by means of a pH stat.
2. Add a quantity of citraconic anhydride to the reaction medium to provide at least a 5–10 molar excess of reagent over the amount of amines to be modified. Even greater molar excesses may be required for total blocking of all the amines of some proteins.
3. React for at least 1–2 hours at room temperature. To assure complete blocking of all amine groups, the reaction may be continued overnight.
4. Remove excess reactants from the citraconylated molecule by dialysis or gel filtration. The efficiency of amine modification may be assessed by use of the TNBS test for amines (Section 4.3, this chapter). A negative test for amines indicates complete modification.

To remove the citraconic modifications and free the amine groups, the protein may be treated in one of two ways:

1. Adjust the pH of the citraconylated molecule to 3.5–4.0 by addition of acid. Incubate at room temperature overnight or for at least 3 hours at 30°C.

   or

2. Treat the citraconylated molecule with 1 M hydroxylamine at pH 10 for 3 hours at room temperature.

Modification of Sulfhydryls with Iodoacetate

Iodoacetate (and bromoacetate) can react with a number of functional groups within proteins: the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and the primary ε-amine group of lysine residues and N-terminal α-amines (Gurd, 1967). The relative rate of reaction with each of these residues is generally dependent on the degree of ionization and thus the pH at which the modification is done. The exception to this is methioninyl thioethers, which react rapidly at nearly all pH values above about 1.7 (Vithayathil and Richards, 1960). The reaction products of these groups with iodoacetate are illustrated in Figure 1.87. The only reaction resulting in one definitive product is that of the alkylation of cysteine sulfhydryls, giving the carboxymethylcysteinyl derivative (Cole *et al.*, 1958). Histidine groups may be modified at either nitrogen atom of its imidazolyl side chain.

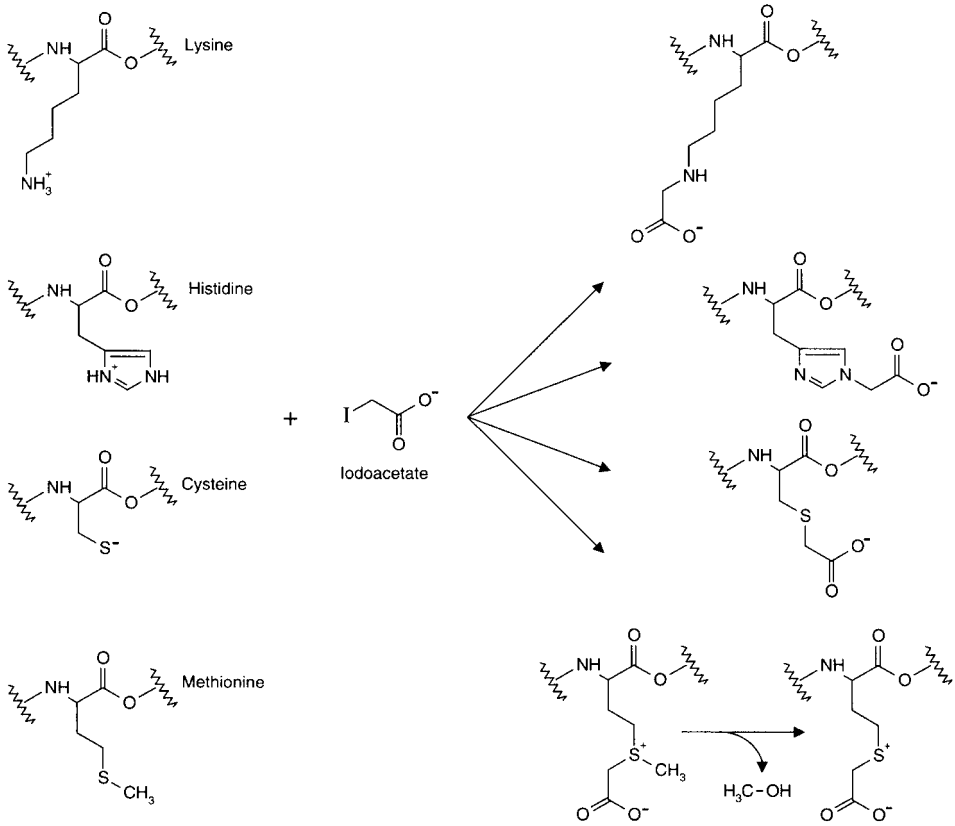
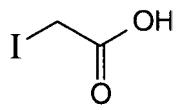


Figure 1.87 Iodoacetate can modify a number of amino acid side chains in proteins, forming alkylated derivatives containing a terminal carboxylate.

Both mono-substituted derivatives and di-substituted products of the imidazole ring are possible (Crestfield *et al.*, 1963). With primary amine groups such as in the side chain of lysine residues, the products of the reaction are the secondary amine (monocarboxymethyllysine) or the tertiary amine derivative (dicarboxymethyllysine). Methionine thioether groups give the most complicated products, some of which rearrange or decompose unpredictably. The only stable derivative of methionine is where the terminal methyl group is lost to form carboxymethylhomocysteine, the same product as the reaction of iodoacetate with homocysteine (Gundlach *et al.*, 1959).



Iodoacetate
MW 185.9

The relative reactivity of α-haloacetates toward protein functionalities is sulfhydryl > imid azolyl > thioether > amine. Among halo derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative

reactivities, but will obviously not create a carboxylate functional group. The acetamide derivatives typically are used only as blocking agents.

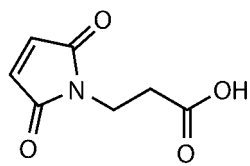
Thus, iodoacetate has the highest reactivity toward sulfhydryl cysteine residues and may be directed specifically for —SH modification. If iodoacetate is present in limiting quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. The specificity of this modification has been used in the design of heterobifunctional crosslinking reagents, where one end of the crosslinker contains an iodoacetamide derivative and the other end contains a different functionality directed at another chemical target (see SIAB; Chapter 5, Section 1.5).

Protocol

1. Dissolve the sulfhydryl-containing protein or macromolecule to be modified at a concentration of 1–10 mg/ml in 50 mM Tris, 0.15 M NaCl, 5 mM EDTA, pH 8.5. EDTA is present to prevent metal-catalyzed oxidation of sulfhydryl groups. The presence of Tris, an amine-containing buffer, should not affect the efficiency of sulfhydryl modification. Not only do amines generally react slower than sulfhydryls, the amine in Tris buffer is of particularly low reactivity. If Tris does pose a problem, however, use 0.1 M sodium phosphate, 0.15 M NaCl, 5 mM EDTA, pH 8.0.
2. Add iodoacetate to a concentration of 50 mM in the reaction solution. Alternatively, add a quantity of iodoacetate representing a 10-fold molar excess relative to the number of —SH groups present. An estimation of the sulfhydryl content in the protein to be modified can be accomplished by performing an Ellman's assay (Chapter 1, Section 4.1). Readjust the pH if necessary. To aid in adding a small quantity of iodoacetic acid to the reaction, a concentrated stock solution may be made in the reaction buffer, the pH re-adjusted, and an aliquot added to the protein solution to give the desired concentration.
3. Mix and react for 2 hours at room temperature. To avoid the possibility of methionine modification, limit the reaction to 30 minutes.
4. Purify the modified protein from excess iodoacetate by dialysis or gel filtration.
5. An Ellman's assay comparing the unmodified protein to the iodoacetylated protein may be done to assess the degree of modification.

Modification of Sulfhydryls with BMPA

BMPA is *N*-β-maleimidopropionic acid (or 3-maleimidopropionic acid), which contains a thiol-reactive maleimide group at one end and a carboxylate group on the other end (Rich *et al.*, 1975; Moroder, 1983, 1987). The compound is the acid precursor to the short, heterobifunctional crosslinker 3-maleimidopropionic acid *N*-hydroxysuccinimide ester (BMPS).



BMPA;
N-β-Maleimidopropionic acid
MW 169.13

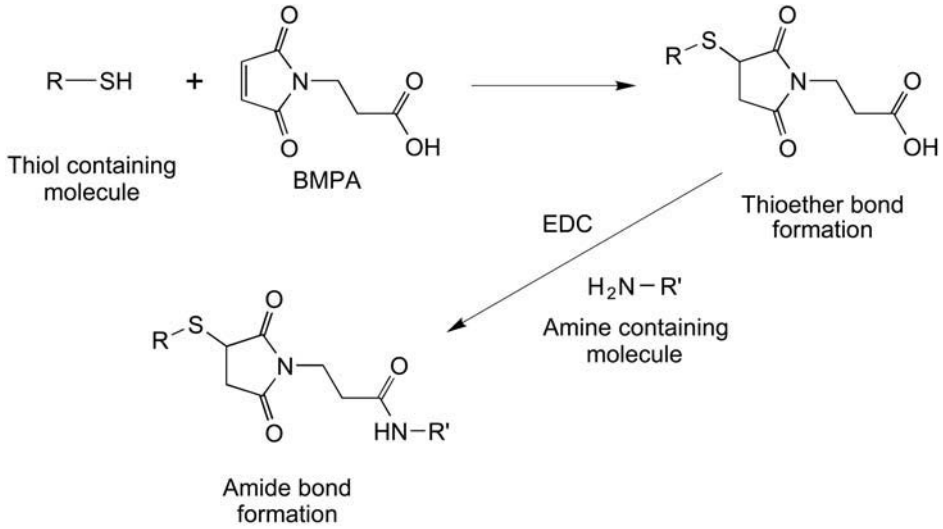


Figure 1.88 The maleimide group of BMPA reacts with a thiol-containing molecule to result in a modification having a terminal carboxylate group. Amine-containing molecules then can be conjugated to the carboxylate using a carbodiimide reaction with EDC.

Like BMPS, BMPA is spontaneously reactive toward sulfhydryls through its maleimide, but unlike BMPS it must be activated using a carbodiimide, such as EDC, to couple to amines or hydrazides through its carboxylic acid end. In its use as a blocking or modification agent for sulfhydryls, BMPA may be reacted with a thiol-containing molecule to form a stable thioether bond. The blocking of thiols takes place in buffered aqueous conditions from slightly acidic to moderately basic pH by addition to the double bond of the maleimide group. The final product, which then contains the short propionic acid spacer, terminates in a negatively charged carboxylate. Thus, thiols can be modified and transformed into carboxylate-containing molecules using this reagent.

BMPA also has been used as a crosslinking agent in a number of applications, including the preparation of peptide–protein conjugates for immunogens Cruz *et al*., to prepare immunomodulating adducts (Gemeiner *et al*., 1992; Cruz *et al*., 2001), in the preparation of novel trifunctional compounds containing the metal-chelating group lysine nitrilotriacetic acid (NTA) for conjugation with His-tagged proteins (Meredith *et al*., 2004), for the immobilization of proteins onto surfaces (Jung and Wilson, 1996), to create thiol-reactive quantum dots for labeling biomolecules (Evident Technologies, Inc., web site protocols, 2005), to form albumin–insulin conjugates for slow-release drugs (Shechter *et al*., 2005), and to synthesize thiol-reactive luminescent chelates for time-resolved fluorescence applications (Chen and Selvin, 1999).

Figure 1.88 shows the reactions of BMPA for the modification of thiols and a subsequent reaction using an EDC-mediated amide bond formation for coupling to its carboxylate end. The maleimide–thiol reaction proceeds at physiologic pH to form a stable thioether linkage with sulfhydryl-containing molecules. The combination of EDC and sulfo-NHS (Chapter 3, Section 1.2) also may be used to form an intermediate sulfo-NHS ester, which can enhance the yield of amide bond formation. Cysteine groups in proteins and peptides may be permanently blocked using this reagent, yielding a modification that terminates in the negatively charged carboxylate.

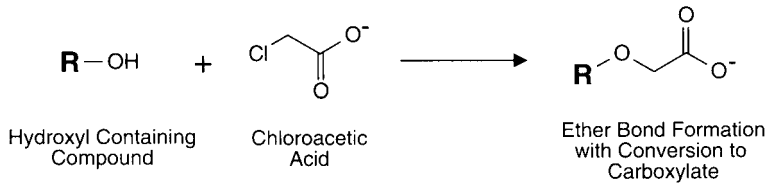


Figure 1.89 Chloroacetic acid can be used to create a carboxylate group from a hydroxyl.

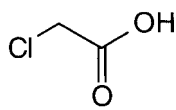
The use of BMPA to block a thiol and create a terminal carboxylate is illustrated in the following protocol. The protocol relates to the modification of proteins, but similar reaction conditions can be used to modify other thiol-containing molecules or surfaces.

Protocol

1. Dissolve a thiol-containing protein in phosphate buffered saline (PBS), pH 6.5–7.5, at a concentration 1–10 mg/ml. Disulfides may be reduced to yield free thiols using DTT, TCEP, or other reducing agents, but reductants containing sulfhydryls should be completely removed by dialysis or desalting prior to reaction with BMPA.
2. Dissolve BMPA in DMSO or DMF to prepare a stock solution at a higher concentration such that adding an aliquot of this solution to the protein solution will result in the desired molar excess of the maleimide over the concentration of thiols present.
3. Add a quantity of BMPA to the protein solution to obtain at least a 5-fold molar excess of maleimide reagent over the amount of thiol present in the protein. The final concentration of organic solvent in the protein solution should not exceed 10 percent to prevent protein precipitation. Mix thoroughly to dissolve.
4. React for 2 hours at room temperature.
5. Purify the modified protein from reactants and reaction by-products by dialysis or gel filtration.

Modification of Hydroxyls with Chloroacetic Acid

Chloroacetic acid can be used to transform a rather unreactive hydroxyl into a carboxylate group that can be used in a variety of conjugation reactions. The reaction proceeds under basic conditions, yielding a stable ether bond terminating in a carboxymethyl group (Figure 1.89) (Plotz and Rifai, 1982; Brunswick *et al.*, 1988). Side reactions will occur with other nucleophiles, such as amines, if they are present in the molecule to be modified. The reagent is used most often to modify pure polysaccharides or hydroxyl-containing polymers that contain no other functionalities.



Chloroacetic Acid
MW 94.47

The following protocol illustrates the modification of a dextran polymer with chloroacetic acid.

Protocol

1. In a fume hood, prepare a solution consisting of 1 M chloroacetic acid in 3 M NaOH.
2. Immediately add dextran polymer to a final concentration of 40 mg/ml. Mix well to dissolve.
3. React for 70 minutes at room temperature with stirring.
4. Stop the reaction by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 N HCl.
5. Remove excess reactants by dialysis.

4.3. Introduction of Primary Amine Groups

Primary amine groups on proteins consisting of N-terminal α-amines and lysine side chain ε-amines are typically present in abundant quantities for modification or conjugation reactions. Occasionally, however, a protein or peptide will not contain sufficient amounts of available amines to allow for an efficient degree of coupling to another molecule or protein. For instance, HRP, a popular enzyme to employ in the preparation of antibody conjugates, only possesses two free amines that can participate in conjugation protocols. Creating additional amines on HRP allows for higher amounts of modification and thus produces more active conjugates.

Other non-protein molecules, such as nucleic acids and oligonucleotides, may not normally possess primary amines of sufficient nucleophilicity to react with common modification reagents. The ability to add amine functionalities to these molecules is sometimes the only route to successful conjugation. Creating amines at specific sites within these molecules allows for site-directed modification at known positions, thus better assuring active conjugates once formed.

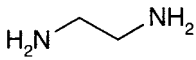
The following reagents and techniques can be used to directly transform carboxylates or sulfhydryls into reactive amine functional groups. In addition, sugars, polysaccharides, or glycan containing macromolecules may be modified to contain amines after mild periodate activation to form aldehyde groups or through modification at the reducing end of a carbohydrate.

Modification of Carboxylates with Diamines

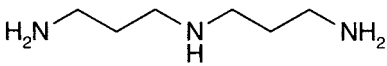
Carboxylic acids may be covalently modified with short compounds containing primary amines at either end to form amide linkages. The result of such alterations is to block the carboxylates and form terminal amino groups. Reacting the diamine in high molar excess assures that only one end of the compound couples to each carboxylate and does not crosslink the molecules being modified. Amide bond formation may be accomplished by several methods including carbodiimide-mediated coupling (Chapter 3, Section 1), active ester intermediates such as *N*-hydroxysuccinimide esters (Chapter 2, Section 1.4), and the use of carbonylating compounds like *N*,*N*′-carbonyldiimidazole (Chapter 3, Section 3). A combination of the water-soluble carbodiimide EDC and sulfo-NHS also is an efficient way of creating amide linkages (Chapter 3, Section 1.2).

Diamines that can be used for aminoalkylation include ethylene diamine, 1,3-diaminopropane, 3,3′-imino*bis*propylamine (also known as diaminodipropylamine), 1,6-diaminohexane, and the Jeffamine-type compounds containing a hydrophilic chain consisting of polyethylene- or polypropylene-oxide (formerly from Texaco Chemical Co., now Huntsman Corporation). Ethylene diamine is perhaps the most popular choice for protein carboxylate modification. Its short chain length assures minimal steric effects and virtually no hydrophobic interactions. Diaminodipropylamine provides a longer spacer arm and has been used extensively as a bridging molecule for coupling carboxylate-containing ligands to insoluble supports (Hermanson *et al.*, 1992). The long hydrocarbon chain of 1,6-diaminohexane, however, may induce hydrophobic effects and probably should be avoided. The longest diamine of the group is the Jeffamine compound. Its chain is extremely hydrophilic and should function as an excellent modifier of carboxylates when a longer spacer is desired.

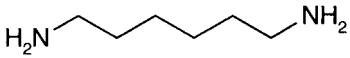
In addition, there are other diamine spacers containing discrete PEG chains available with one end blocked using either a t-BOC group or a CBZ-amido group (Quanta BioDesign). These diamine spacers are extremely hydrophilic due to the presence of a PEG_3 or PEG_{11} cross-bridge units. Since one end of these compounds is masked with a reversible blocking group commonly used in peptide synthesis, the free amine end can be conjugated to a carboxylic acid without the possibility of crosslinking. The blocked end then can be removed with TFA (for t-BOC groups) or by reduction using hydrogen in the presence of a Pd/C catalyst (for CBZ-amido groups). This type of deblocking reaction only should be used with molecules that can tolerate these conditions, such as organic molecules and short peptides. Complex proteins, however, may be denatured or loose activity under those conditions.



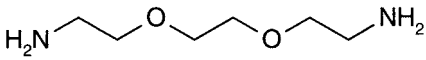
Ethylenediamine
MW 60



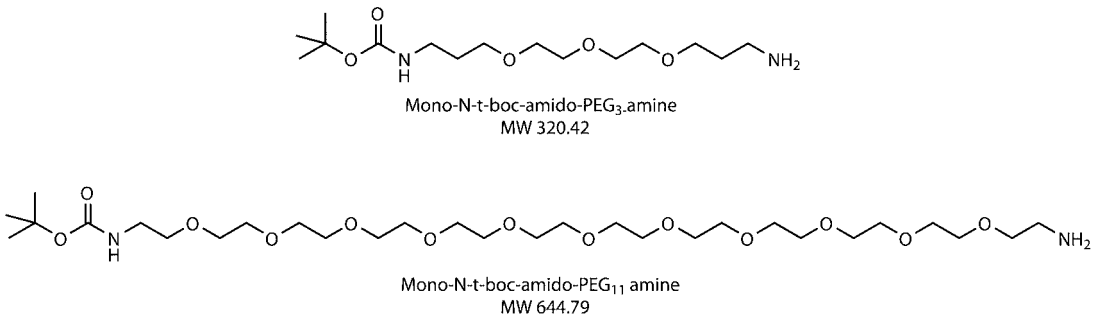
3,3'-Iminobispropylamine
(diaminodipropylamine)
MW 131



1,6-Diaminohexane
MW 116



Jeffamine EDR-148
MW 148



Diamine modification of proteins can have dramatic effects on the net charge of the molecule, usually significantly raising the pI from the native state. The amide linkage eliminates the negative potential of the carboxylate and the terminal amine adds the potential for a positive charge. Thus, diamine modification may have the net effect of changing the overall charge by plus two for every carboxylate residue coupled. Proteins heavily modified with diamines may exhibit vital changes in activity due to the alteration of microenvironmental charge at each site of modification. In some cases, native conformation may be changed and activity completely lost.

Raising the pI of macromolecules also can significantly alter the immune response toward them upon *in vivo* administration. Cationized proteins (those modified with diamines to increase their net charge or pI) are known to generate an increased immune response compared to their native forms (Muckerheide *et al*., 1987a, b; Apple *et al*., 1988; Domen *et al*., 1987; Domen and Hermanson, 1992). The use of cationized BSA as a carrier protein for hapten conjugation can result in a dramatically higher antibody response toward a coupled hapten (Chapter 19).

The following protocol using the carbodiimide EDC is an efficient way of modifying protein carboxylates with diamines to either increase the amount of amines present for further conjugation or to create a cationized protein having an increased net charge (Figure 1.90). Note that

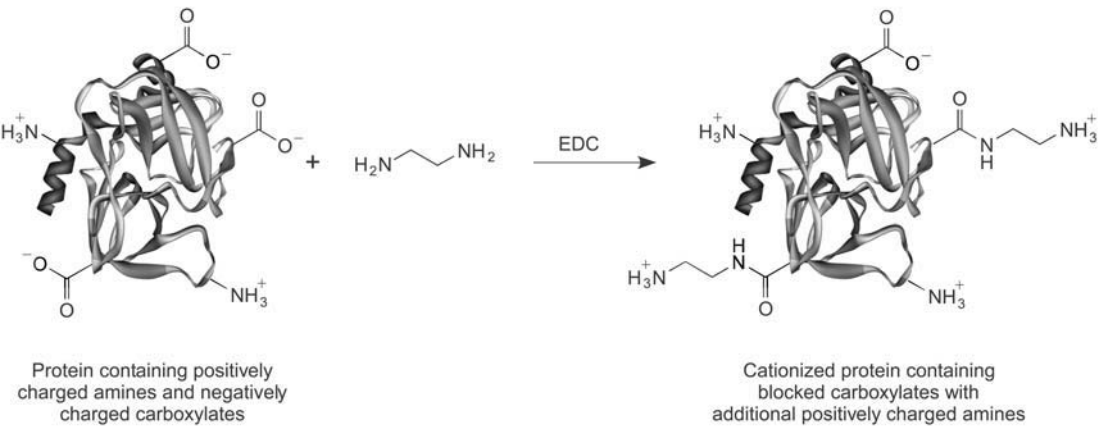


Figure 1.90 Cationization of protein molecules can be done using ethylene diamine to modify carboxylate groups using a carbodiimide reaction process.

glycoproteins containing sialic acid may be modified at this sugar's —COOH group in addition to coupling at C-terminal, aspartic acid, and glutamic acid functions on the polypeptide chain. Other carboxylate-containing macromolecules may be modified using this procedure as well.

Protocol

1. Dissolve the protein to be modified at a concentration of 1–10 mg/ml in 0.1 M MES, pH 4.7 (coupling buffer). Other buffers may be used as long as they don't contain groups that can participate in the carbodiimide reaction. Avoid carboxylate- or amine-containing buffers such as citrate, acetate, glycine, or Tris. Higher pH conditions may be used up to about pH 7.5 (in sodium phosphate buffer) without severely affecting the yield of modification. The protein in solid form also may be added directly to the diamine solution prepared in (2).
2. Dissolve the diamine chosen for modification at a concentration of 1 M made up in the coupling buffer. If a free-base form of diamine is used, then the solution will become highly alkaline upon dissolution. This operation also will generate heat—the solution process being highly exothermic. The easiest way to dissolve such a diamine is to initially add the correct amount to a beaker containing a quantity of crushed ice equal to the final solution volume desired. The ice should be made from deionized water or the equivalent to maintain purity. All operations should be done in a fume hood. Next, add an equivalent weight of concentrated HCl and mix. As the mixing becomes complete, the ice will almost totally melt and provide nearly the correct final solution volume. Finally, add an amount of MES buffer salt to bring its concentration to 0.1 M, and adjust the solution pH to 4.7. In some cases, the dihydrochloride form of the diamine is commercially available and can be used to avoid such unpleasant pH adjustments. For instance, ethylene diamine dihydrochloride is available from Aldrich. It can be added to the 0.1 M MES buffer without a significant change in pH.
3. Add the protein solution to an equal volume of diamine solution and mix. Alternatively, the solid protein can be dissolved directly in the diamine solution (after pH adjustment) at the indicated concentration.
4. Add EDC hydrochloride; Thermo Fisher to a final concentration of 2 mg/ml in the reaction solution. To aid in the addition of a small amount of EDC, a higher concentration stock solution may be prepared in water and an aliquot added to the reaction to give the proper concentration. Since EDC is labile in aqueous solutions, the stock solution must be made quickly and used immediately.
5. React for 1–2 hours at room temperature.
6. Purify the modified protein by extensive dialysis against 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) or another suitable buffer.

The changes that occur in the pI of a protein modified with diamines may be assessed by isoelectric focusing or by general electrophoresis based upon relative migration due to charge. A cationized protein will possess a higher pI value or migrate further toward the anode than its native form. Using the above protocol typically alters the net charge of BSA from a native pI of 4.9 to the highly basic range of pI 9.5 to over pI 11.0.

Modification of carboxylate groups with diamines also may be done in organic solvent for those molecules insoluble in aqueous buffers. Some peptides are quite soluble in solvents such as DMF and DMSO, but relatively insoluble in water. Such molecules may be reacted in these

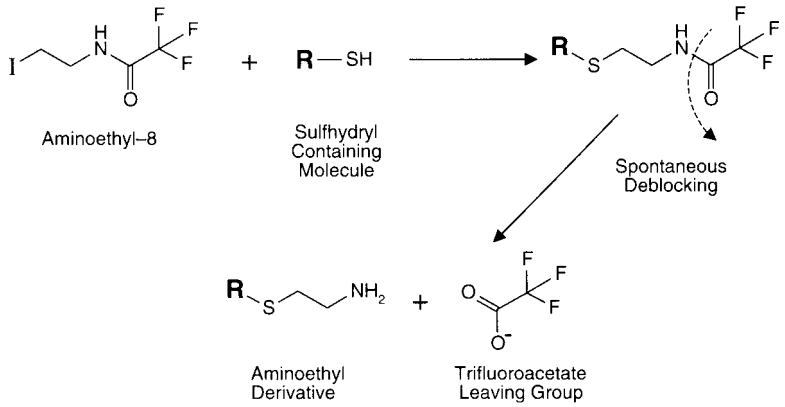
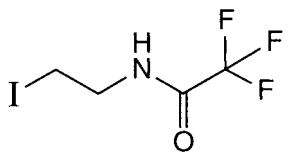


Figure 1.91 Aminoethyl-8 can be used to transform a sulfhydryl group into an amine. The intermediate spontaneously undergoes deblocking to release the primary amine group.

solvents with the carbodiimide DCC (dicyclohexyl carbodiimide) using the same basic reactant ratios as given above for EDC in aqueous solutions (Chapter 3, Section 1.4).

Modification of Sulfhydryls with N-(β-Iodoethyl)trifluoroacetamide [Aminoethyl-8]

The conversion of sulfhydryl groups on cysteine residues or other molecules to amine-containing groups may be accomplished by aminoethylation with *N*-(β-iodoethyl) trifluoroacetamide (Schwartz *et al.*, 1980). The haloalkyl group specifically reacts with sulfhydryls to form the aminoalkyl derivative in one step. Under the conditions of the reaction, the trifluoroacetate amine-protecting group spontaneously hydrolyzes to expose the free primary amine without the need for a secondary deblocking step (Figure 1.91). This reagent is commercially available from Thermo Fisher Chemical under the name Aminoethyl-8™.



Aminoethyl–8™ Reagent
N-(iodoethyl)trifluoroacetamide
MW 267

Aminoethyl-8 has an advantage over ethylenimine modification (see next section), due to the potential polymerization of ethylenimine in aqueous solutions. Such polymers are highly cationic and may nonspecifically block the protein. The specificity of Aminoethyl-8 for sulfhydryls makes it an optimum choice for modification.

For small molecules containing sulfhydryls or for low-molecular-weight peptides containing cysteine residues, modification may proceed without deforming agents. However, for intact proteins containing both disulfides and free sulfhydryls, a denaturant and a disulfide reducing

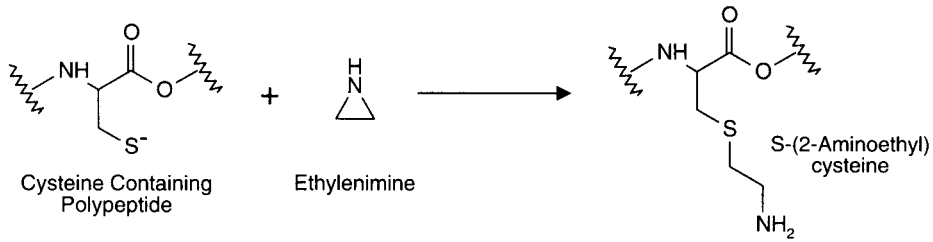


Figure 1.92 The small compound ethylenimine can react with sulfhydryls to form aminoethyl derivatives.

agent may be required to open buried or structurally inaccessible groups if complete modification is desired.

Protocol

1. Dissolve the protein to be aminoalkylated at a concentration of 1–10 mg/ml in 6 M guanidine hydrochloride, 0.2 M *N*-ethylmorpholine acetate, pH 8.1. All water used in preparing buffers should be deoxygenated by boiling followed by cooling and bubbling with nitrogen. Small molecules that don't require denaturants to expose internal disulfides or sulfhydryls may be modified without using guanidine treatment.
2. Add DTT to obtain a 20-fold molar excess over the amount of disulfides present.
3. React for 4 hours at room temperature, maintaining a blanket of nitrogen over the solution.
4. Adjust the pH to 8.6 with NaOH, and heat the solution to 50°C.
5. Add a quantity of Aminoethyl-8 in methanol to equal a 25-fold molar excess over the amount of sulfhydryl present (including the amount of DTT added). The solution in methanol should be made concentrated enough so only a small amount of methanol has to be added to the reaction solution (i.e., no more than 10 percent of the final volume). A second addition of modifying agent may be made after 1 hour to drive the reaction more completely toward total —SH aminoalkylation.
6. React for 3 hours at 50°C.
7. Purify the modified protein or other macromolecules by gel filtration or dialysis. Occasionally, complete modification with Aminoethyl-8 will cause precipitation of the protein.

Modification of Sulfhydryls with Ethylenimine

The cyclic compound ethylenimine reacts with protein sulfhydryl groups causing ring opening and forming the aminoalkyl derivative, *S*-(2-aminoethyl)cysteine (Raftery and Cole, 1963, 1966) (Figure 1.92). Under physiological conditions ethylenimine is virtually specific for sulfhydryls with no cross-reactivity toward other protein functionalities. At acid pH, a small degree of reactivity occurs with methionine residues, forming *S*-(2-aminoethyl)methionine sulfonium ion (Schroeder *et al.*, 1967). Since aminoethylated cysteine groups resemble the side-chain structure of lysine residues, except for the replacement of one methylene group with a thioether, these modifications make them susceptible to tryptic hydrolysis, although at an abbreviated rate (Plapp *et al.*, 1967; Wang and Carpenter, 1968).

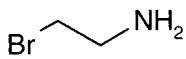
Ethylenimine
MW 43

Ethylenimine may be used to introduce additional sites of tryptic cleavage for protein structural studies. In this case, complete sulfhydryl modification is usually desired. Proteins are treated with ethylenimine under denaturing conditions (6–8 M guanidine hydrochloride) in the presence of a disulfide reductant to reduce any disulfide bonds before modification. Ethylenimine may be added directly to the reducing solution in excess (similar to the procedure for Aminoethyl-8 described previously) to totally modify the —SH groups formed.

The disadvantage of using ethylenimine for protein modification stems from the fact that in the presence of water, slow formation of polyethylenimine occurs. The polymer is highly positively charged at physiological pH and can interact strongly with protein molecules, masking sites of potential sulfhydryl modification. Also, the polymer may have terminal aziridine residues (Chapter 2, Section 2.3), making it reactive and potentially forming a covalent attachment with the protein (Dermer and Ham, 1969).

Modification of Sulfhydryls with 2-Bromoethylamine

2-Bromoethylamine may undergo two reaction pathways in its modification of sulfhydryl groups in proteins (Figure 1.93). In the first scheme, the thiolate anion of cysteine attacks the No. 2 carbon of 2-bromoethylamine to release the halogen and form a thioether bond (Lindley, 1956). This straightforward reaction mechanism is similar to the modification of sulfhydryls with iodoacetate (Chapter 1, Section 4.2). In a two-step, secondary process, 2-bromoethylamine is converted under alkaline conditions to the cyclic ethylenimine derivative by the intramolecular attack of its primary amine on the No. 2 carbon, causing release of the halogen and ring formation (Cole, 1967). Ethylenimine then goes on to react with the sulfhydryl to form the aminoalkylated derivative (as described in the previous section). The two-step reaction is slower than direct aminoalkylation by either 2-bromoethylamine or ethylenimine.



2-Bromoethylamine
MW 123.92

Protocol

1. Dissolve the protein or peptide to be aminoalkylated at cysteine sulfhydryls in 0.5 M sodium carbonate. If cystine disulfides are present, add a 10- to 25-fold molar excess of DTT to fully reduce them to free sulfhydryls.
2. Add a quantity of 2-bromoethylamine to obtain a 10-fold molar excess over the number of sulfhydryls present in the sample, including any added DTT.

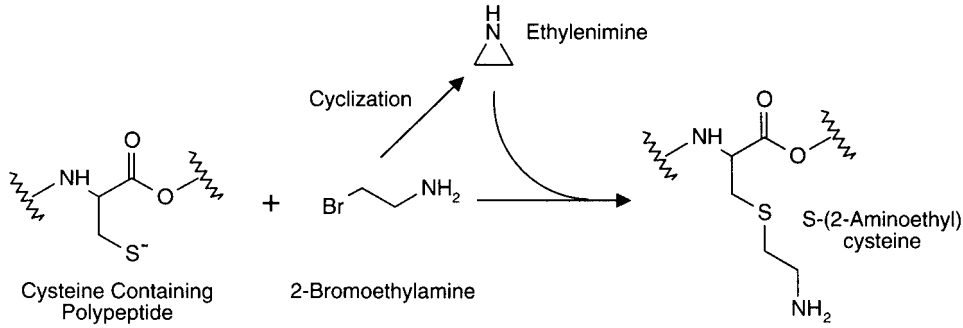
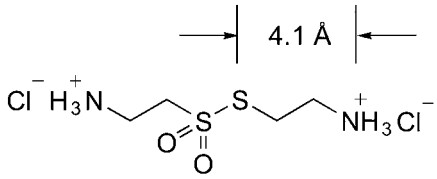


Figure 1.93 2-Bromoethylamine can be used to transform a thiol into an amine. The reaction may proceed through the intermediate formation of ethylenimine, yielding an aminoethyl derivative.

3. React overnight at room temperature.
4. Purify the modified protein by gel filtration or dialysis.

Modification of Sulfhydryls with 2-Aminoethyl-2′-aminoethanethiolsulfonate

Thiolsulfonate-containing compounds can react with thiols with release of the sulfonate end of the molecule to yield disulfide derivatives. The modification reagent 2-aminoethyl-2′-aminoethanethiolsulfonate, or AEAETS, reacts with a sulfhydryl with release taurine (2-aminoethanesulfonate) to form a 2-aminoethyl-dithiol derivative (Figure 1.94). AEAETS can be used to block cysteine residues in proteins and form derivatives containing positively charged amines.



AEAETS
2-Aminoethyl-2'-
aminoethanethiolsulfonate
dihydrochloride
Mol. Wt.: 257.20

The basic reactions of thiolsulfonates have been known for sometime (Field *et al.*, 1961, 1964), but more recently, they have been applied to the study of protein interactions by site-directed modification of native cysteines or through modification of cysteines introduced at particular points in proteins by mutagenesis. Such studies have yielded insights into the structure and binding site characteristics of proteins (Kirley, 1989). Pascual *et al.* (1998) used AEAETS to probe the acetylcholine receptor from the extracellular side of the membrane in order to investigate the molecular accessibility and electrostatic potential within the open and closed channel.

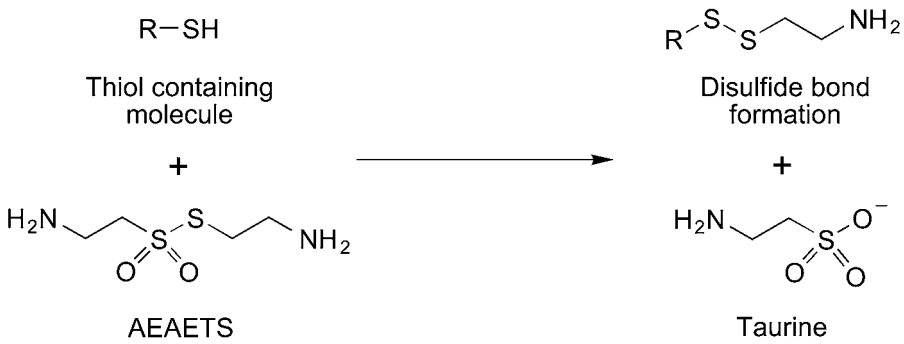


Figure 1.94 AEAETS reacts with thiol groups to form a disulfide modification that terminates in a primary amine.

The AEAETS reaction with thiols is similar to that of sodium tetrathionate (Section 5.2, this chapter) and the methanethiosulfonate (MTS) or disulfide exchange compounds described in Chapter 2, Section 2.6. All of these reagents form disulfides upon reaction with sulfhydryls, and the modifications subsequently can be reversed using disulfide reducing agents, like DTT or TCEP. AEAETS is moisture sensitive and hydrolyzes slowly in aqueous solution, cleaving to release mecaptoethylamine and taurine. Also, avoid contact with oxidizing agents, such as peroxide, as these will oxidatively cleave and inactivate the reagent.

AEAETS is freely soluble in aqueous buffers and in DMF or DMSO to about 100 mg/ml. A stock solution may be made in organic solvent and a small aliquot transferred to a buffered reaction medium to initiate the reaction. The reaction of AEAETS with thiols takes place rapidly (within minutes) provided the group is accessible. Since cysteine is the least accessible amino acid in proteins (Section 1.1, this chapter), globular proteins may contain sulfhydryls that are buried or not fully accessible to the surrounding aqueous environment, and these may react slowly or not at all. The modification of cysteine thiols may be done in 10 mM HEPES, pH 7.5, and containing 150 mM NaCl using 10–100 μM AEAETS in the final reaction medium.

Modification of Carbohydrates with Diamines

Carbohydrates or oligosaccharides may be modified to contain primary amino groups by selective reaction with a diamine compound. Several reaction pathways may be used to accomplish this modification. In some cases, a particular carbohydrate may contain sugar residues that possess potential amine coupling groups without prior derivatization to form such functionalities. For example, if carboxylate-containing sugars are present like sialic or uronic acid (Figure 1.95), then direct modification with a diamine is possible using the carbodiimide coupling protocol described previously in this section.

If carboxylates are lacking in the carbohydrate molecule, then indigenous hydroxyls may be utilized to create aldehydes for coupling diamines by one of two routes. The simplest method of creating amine-reactive groups in sugar molecules is by oxidation using sodium periodate (Section 4.4, this chapter). Periodic acid cleaves adjacent hydroxyls to form highly reactive aldehyde groups (Rothfus and Smith, 1963). At a concentration of 1 mM in the cold, sodium periodate specifically cleaves only at the adjacent hydroxyls between the Nos. 7, 8, and 9 carbon atoms of sialic acid residues (Van Lenten and Ashwell, 1971; Wilchek and Bayer, 1987). The product is the formation of one aldehyde group on the No. 7 carbon and liberation of two molecules

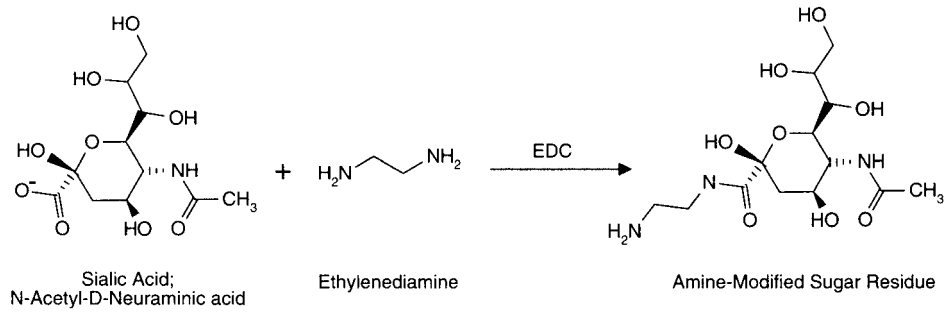


Figure 1.95 Carboxylate-containing sugars may be modified with diamines using a carbodiimide-mediated reaction to create available amine groups for subsequent conjugation.

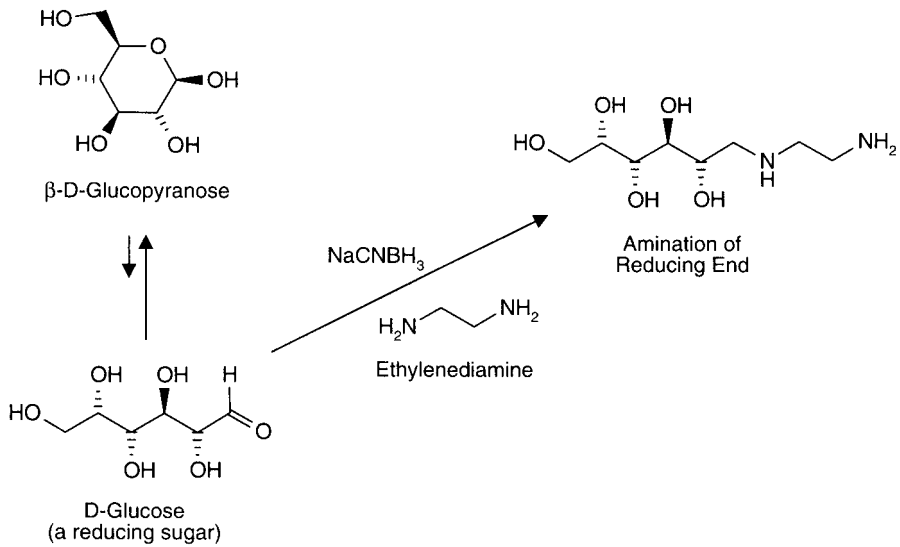


Figure 1.96 Reducing sugars may be aminated with diamines in the presence of sodium cyanoborohydride to produce amine modifications.

of formaldehyde. The sialic acid aldehyde then can be coupled with diamines by Schiff base formation and reductive amination (Chapter 2, Section 5.3 and Chapter 3, Section 4).

Oxidation of polysaccharides using 10 mM or greater concentrations of sodium periodate results in the cleavage of adjacent diol-containing carbon–carbon bonds on other sugars besides just sialic acid residues. Glycoproteins and polysaccharides may be modified using this procedure to form multiple formyl functionalities for coupling diamines or other amine-containing molecules.

In some instances, reducing sugars are present that can be reductively aminated without prior periodate treatment. A reducing end of a monosaccharide, a disaccharide, or a polysaccharide chain may be coupled to a diamine by reductive amination to yield an aminoalkyl derivative bound by a secondary amine linkage (Figure 1.96). Also see Section 4.6, this chapter, for an extensive discussion on carbohydrate modification techniques.

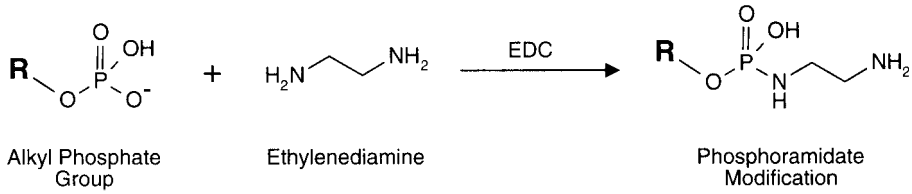


Figure 1.97 Phosphate groups may be modified to possess amines by a carbodiimide reaction in the presence of a diamine.

An alternative to the use of chemical means to create formyl groups is the specific modification afforded by sugar oxidases (Section 4.4, this chapter). For instance, galactose oxidase may be reacted with a carbohydrate-containing terminal D-galactose or *N*-acetyl-D-galactosamine residues to transform the C-6 hydroxyl group into an aldehyde (Avigad *et al.*, 1962). Subsequent reaction with a diamine yields the desired amine modification.

The appropriate protocols for diamine modification of various carbohydrate or glycoprotein derivatives may be found in the indicated sections.

Modification of Alkylphosphates with Diamines

Alkylphosphate groups can be made to react with diamines to form aminoalkylphosphoramidate modifications. The primary amine thus formed then may be used to conjugate with other molecules containing amine-reactive groups. In this sense, DNA or RNA may be modified with a diamine at the 5′-phosphate group mediated by a carbodiimide reaction. *N*-substituted carbodiimides can react with a phosphate group to form highly reactive phosphodiester derivatives that are extremely short-lived in aqueous solution (Chapter 3, Section 1) (Figure 1.97). This active species then can react with a nucleophile such as a primary amine to form a phosphoramidate bond (Chu *et al.*, 1986). The process is analogous to the activation of a carboxylate by a carbodiimide with subsequent coupling to an amine-containing molecule to form an amide linkage (Williams and Ibrahim, 1981).

In most procedures, the water-soluble carbodiimide EDC hydrochloride is the most effective mediator of this reaction. Both EDC and its reaction by-products are fully soluble in aqueous buffers and can be easily separated from the modified aminoalkylphosphate (Chapter 3, Section 1.1).

In some methods, the reaction is carried out in a two-step process by first forming an intermediate, reactive phosphorylimidazolide by EDC conjugation in an imidazole buffer. Next, the diamine, in this case cystamine, is reacted with the activated oligonucleotide, causing the imidazole to be replaced by the amine and creating a phosphoramidate linkage (Chu *et al.*, 1986). An easier protocol was described by Ghosh *et al.* (1990) in which the oligo, cystamine, and EDC were all reacted together in an imidazole buffer. A modification of this method is described in Chapter 27, Section 2.1.

Modification of Aldehydes with Ammonia or Diamines

Aldehyde groups can be converted into terminal amines by a reductive amination process with ammonia or a diamine compound. The reaction proceeds by initial formation of a Schiff base

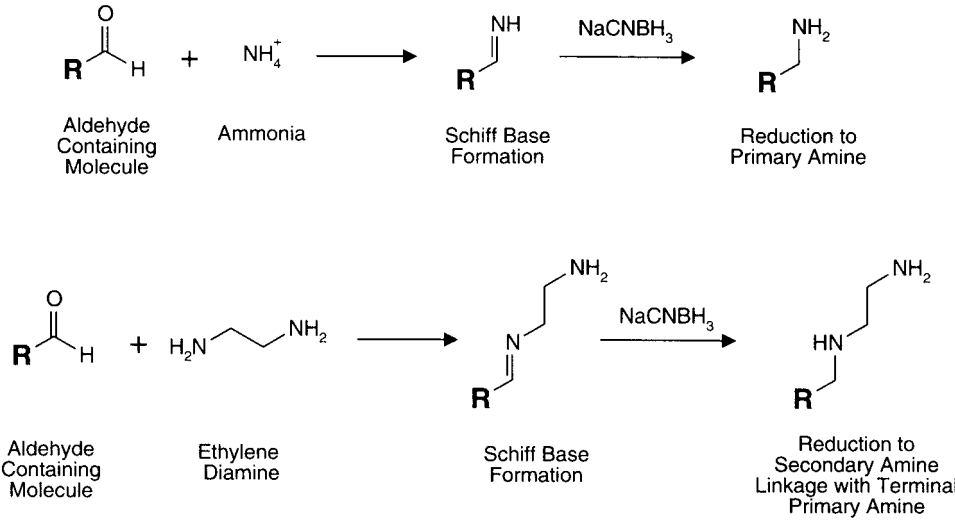


Figure 1.98 Aldehydes may be transformed into primary amines by reaction with ammonia or a diamine in the presence of a reducing agent.

interaction—a dehydration step yielding an imine derivative. Reduction of the Schiff base with sodium cyanoborohydride or sodium borohydride produces the primary amine (in the case of ammonia) or a secondary amine derivative terminating in a primary amine (for a diamine compound) (Figure 1.98).

This simple strategy can be used to add amine residues to polysaccharide molecules after formation of aldehydes by periodate or enzymatic oxidation (Section 4.4, this chapter). Thus, glycoconjugates or carbohydrate polymers such as dextran may be derivatized to contain amines for further conjugation reactions.

The reaction occurs rapidly at alkaline pH (7–10), with higher pH values resulting in better yields due to faster Schiff base formation. To assure complete conversion of available aldehydes to amines, add the ammonia or diamine compound to the reaction in at least a 10-fold molar excess over the expected number of formyl groups present. Diamines that are commonly used for this process include ethylene diamine, diaminodipropylamine (3,3′-iminobispropylamine), 1,6-diaminohexane, the Jeffamine derivative EDR-148 containing a hydrophilic, 10-atom chain (Texaco Chemical Co.), and other PEG-based diamines having one end masked with a reversible blocking agent (see previous discussion of discrete PEG diamines in this section).

Introduction of Arylamines on Phenolic Compounds

Compounds having phenol ring structures, such as tyrosine residues in proteins, often can be derivatized to contain aromatic amine groups through a two-stage reaction process. First, the phenolic ring is nitrated with tetranitromethane in aqueous solution to add a nitro group *ortho* (or *para*, if available) to the hydroxyl. This type of modification can be used to detect tyrosine residues by the strong absorptivity of the unprotonated (at pH 9), 3-nitrophenolate ring at 428 nm (extinction coefficient = 4,200 $M^{-1}cm^{-1}$) (Sokolovsky *et al.*, 1967). The method has

been used to quantify the tyrosine content in porcine trypsinogens and trypsins and to modify a variety of other proteins (Vincent *et al.*, 1970; Lundblad, 1991).

Nitration of the tyrosine rings in the four binding pockets of avidin or streptavidin can be done to increase the steric hinderance within the biotin binding sites (Morag *et al.*, 1996). This process yields chromogenic proteins that have reduced binding affinity for biotin, thus allowing elution of biotinylated molecules under mild conditions.

The nitrophenol group of nitrated molecules also may be reduced to an aminophenyl derivative in alkaline conditions with the use of sodium dithionite (sodium hydrosulfite, $Na_2S_2O_4$) (Pojer, 1979). The amine then can be used to conjugate with an amine-reactive crosslinking reagent to label peptides or proteins at their tyrosine side chains. In addition, this strategy can be a route to creating modifiable amine groups on aromatic molecules other than just tyrosine. For instance, the Bolton–Hunter reagent (Chapter 12, Section 5.1) can be used to modify amine groups on proteins, leaving a phenolic end that is typically used as a site for radioiodination. However, such a derivative also could be used to create an arylamine for further transformation into a highly reactive diazonium group for coupling to tyrosines or phenolic functionalities in other molecules (Figure 1.99) (Chapter 2, Section 6.1).

Protocol

1. Dissolve the protein containing tyrosine residues (or another phenolic macromolecule) in 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, at a concentration of 2–4 mg/ml.

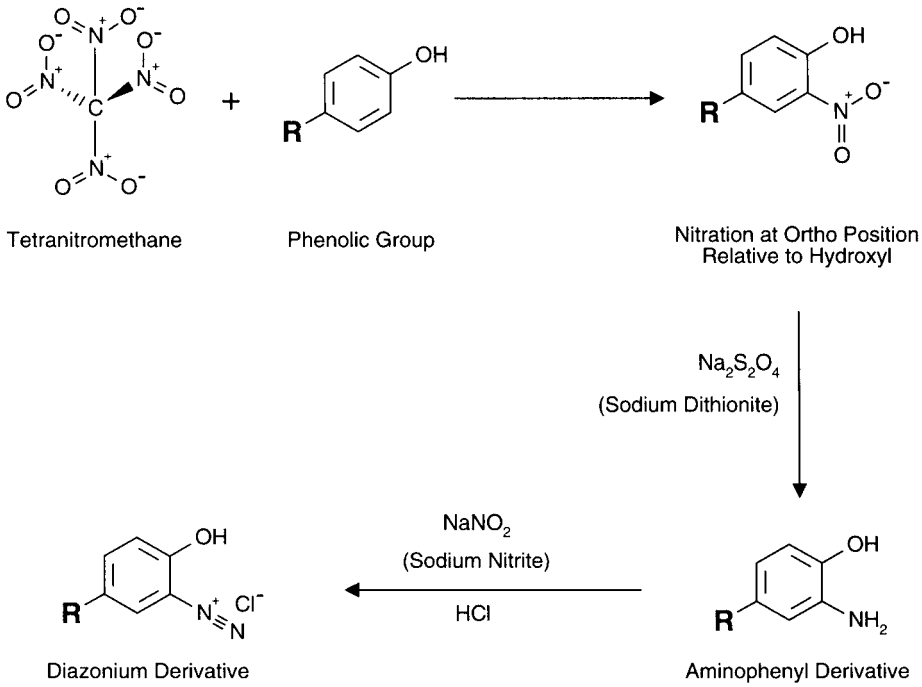


Figure 1.99 Phenolic compounds, such as the side chain of tyrosine residues, may be modified to contain an amine group by nitration followed by reduction to the aminophenyl derivative.

2. With stirring, add to each ml of the protein solution, 20 µl of 0.15 M tetranitromethane in 95 percent ethanol (Sigma). Make the addition in small aliquots if more than several milliliters of solution are to be derivatized. *Note*: All operations with tetranitromethane should be done in a fume hood with extreme care, as this compound is sensitive to heat, friction, and shock or impact.
3. React for 1 hour at room temperature.
4. Quench the reaction by immediate gel filtration using a column of Sephadex G-25 (Pharmacia). Equilibrate the column and perform the chromatography using 0.2 M sodium borate, pH 9.0, so that the protein will be at the proper pH for the reduction step. After the separation, a determination of the modification level may be done by measuring its absorbance at 428 nm.
5. Add sufficient sodium dithionite to bring the final concentration in the reaction medium to 0.1 M.
6. React for 1 hour at room temperature.
7. Purify the aminophenyl derivative by gel filtration or dialysis.

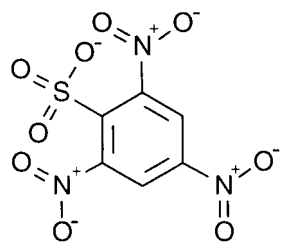
The formation of a diazonium group from the arylamine derivative can be done by treatment with sodium nitrite in HCl (see Protocol in Chapter 9, Section 6).

Amine Detection Reagents

There are several methods available for the detection or measurement of amine groups in proteins and other molecules. Accurate determination of target amine groups in molecules before or after modification may be important for assessing reaction yield or suitability for subsequent crosslinking procedures. The following methods use commercially available reagents and are easily employed to detect primary amines with simple spectrophotometric measurement.

TNBS

Molecules containing primary amines or hydrazide groups can react with 2,4,6-trinitrobenzenesulfonate (TNBS) to form a highly chromogenic derivative (Figure 1.100). This reaction may be used to assay the amine content of compounds by measuring the absorbance of the orange-colored product at 335 nm.



TNBS;
Trinitrobenzene sulfonic acid
MW 293

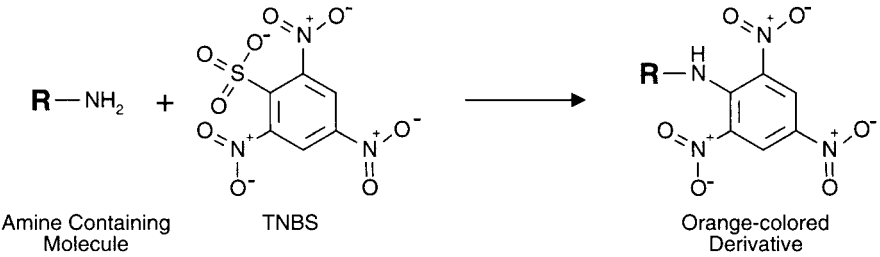


Figure 1.100 TNBS may be used to detect or quantify amine groups through the production of a chromogenic derivative.

TNBS has been used to measure the free amino groups in proteins (Habeeb, 1966; Kakade and Liener, 1969), as a qualitative check for the presence of amines, sulfhydryls, or hydrazides (Inman and Dintzis, 1969), and to specifically determine the number of ε-amino groups of L-lysine in carrier proteins (Sashidhar *et al.*, 1994).

The following protocol may be used for the measurement of amines in soluble molecules, such as proteins or other macromolecules.

Protocol

1. Dissolve or dialyze the molecule to be assayed into 0.1 M sodium bicarbonate, pH 8.5, at a concentration of 20–200 μg/ml (for large molecules like proteins) or 2–20 μg/ml (for small molecules like amino acids).
2. Dissolve TNBS in 0.1 M sodium bicarbonate, pH 8.5, at a concentration of 0.01 percent (w/v). Prepare fresh. *Note*: TNBS may be prepared as a stock solution in ethanol at a concentration of 1.5 percent. This solution is stable to long-term storage and may be diluted as needed in the bicarbonate buffer to the required concentration.
3. Add 0.5 ml of TNBS solution to 1 ml of each sample solution. Mix well.
4. Incubate at 37°C for 2 hours.
5. Add 0.5 ml of 10 percent SDS and 0.25 ml of 1 N HCl to each sample.
6. Measure the absorbance of the solutions at 335 nm. Determination of the number of amines present in a particular sample may be done by comparison to a standard curve generated by use of an amine-containing compound (i.e., an amino acid) dissolved at a series of known concentrations in the bicarbonate sample buffer and assayed under identical conditions.

OPA

OPA (*o*-phthaldialdehyde) is an amine detection reagent that reacts in the presence of 2-mercaptoethanol to generate a fluorescent product (for preparation, see Section 4.1, 2-mercaptoethanol, this chapter) (Figure 1.101). The resultant fluorophore has an excitation wavelength of 360 nm and an emission point at 455 nm. OPA can be used as a sensitive detection reagent for the HPLC separation of amino acids, peptides, and proteins (Fried *et al.*, 1985). It is also possible to measure the amine content in proteins and other molecules using a test-tube or microplate format assay with OPA. Detection limits are typically in the μg/ml range for proteins.

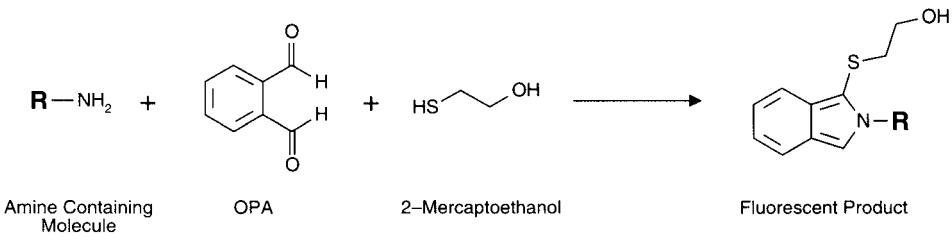


Figure 1.101 OPA reacts with amines to form a fluorescent product.

Protocol

1. Prepare a series of standards, preferably consisting of serial dilutions of the substance to be measured, dissolved in water or non-amine-containing buffer. The concentration range of the standards can be anywhere between about 500 ng/ml and 1 mg/ml.
2. Prepare the samples dissolved in water or non-amine-containing buffer at an expected concentration level that falls within the standard curve range. The assay can tolerate the presence of most buffer salts, denaturants, and detergents. However, the standard curve should be run in the same buffer environment as the samples to obtain consistent response.
3. To a set of labeled tubes, add 2 ml of OPA reagent (Thermo Fisher) and 200 µl of the appropriate standard or sample. Mix well. If using a microplate format, scale back these quantities 10-fold to fit in the microwells.
4. Measure the fluorescence of each sample and standard using an excitation wavelength of 360 nm and an emission wavelength of 436 nm (or using a filter close to the 436–455 nm range).
5. Determine the concentration of the samples by comparison to the standard curve. Since the assay measures the presence of amine groups, the results may be correlated to the relative amount of amines available.

4.4. Introduction of Aldehyde Residues

The formation of an aldehyde group on a macromolecule can produce an extremely useful derivative for subsequent modification or conjugation reactions. In their native state, proteins, peptides, nucleic acids, and oligonucleotides contain no naturally occurring aldehyde residues. There are no aldehydes on amino acid side chains, none introduced by post-translational modifications, and no formyl groups on any of the bases or sugars of DNA and RNA. To create reactive aldehydes at specific locations within these molecules opens the possibility of directing modification reactions toward discrete sites within the macromolecule.

There are two basic ways of introducing aldehyde residues in biological macromolecules: (1) oxidation of carbohydrates- or molecules-containing diols and (2) modification of available amino groups with reagents that contain or produce aldehydes. In both cases, aldehydes can be created that will allow easy conjugation to amine-containing molecules by Schiff base formation and reductive amination (Chapter 2, Section 5.3 and Chapter 3, Section 4). The following sections describe these methods.

Periodate Oxidation of Glycols and Carbohydrates

Carbohydrates and other biological molecules that contain polysaccharides, such as glycoproteins, can be specifically modified at their sugar residues to produce reactive formyl functionalities. With proteins, this method often allows modification to occur only at specific locals, usually away from critical active centers or binding sites.

Periodate oxidation is perhaps the simplest route to transforming the relatively unreactive hydroxyls of sugar residues into amine-reactive aldehydes. Periodate cleaves carbon–carbon bonds that possess adjacent hydroxyls, oxidizing the —OH groups to form highly reactive aldehydes (Bobbit, 1956; Rothfus and Smith, 1963). Terminal cis-glycols result in the loss of one carbon atom as formaldehyde and the creation of an aldehyde group on the former No. 2 carbon atom. Varying the concentration of sodium periodate during the oxidation reaction gives some specificity with regard to what sugar residues are modified. Sodium periodate at a concentration of 1 mM at near 0°C specifically cleaves only at the adjacent hydroxyls between carbon atoms 7, 8, and 9 of sialic acid residues (Van Lenten and Ashwell, 1971; Wilchek and Bayer, 1987). The product is the formation of one aldehyde group on the No. 7 carbon and liberation of two molecules of formaldehyde (Figure 1.102).

Since sialic acid is a frequent terminal sugar constituent of the polysaccharide trees on glycoproteins, this method selectively forms reactive aldehydes on the most accessible parts for subsequent modifications. The carbohydrate polymer of a protein provides a long spacer arm that can be used to conjugate another large macromolecule, such as a second protein, with little steric problems.

Oxidation of polysaccharides using 10 mM or greater concentrations of sodium periodate at room temperature results in the cleavage of adjacent hydroxyl-containing carbon–carbon bonds on other sugars besides just sialic acid residues (Lotan *et al.*, 1975). High concentrations

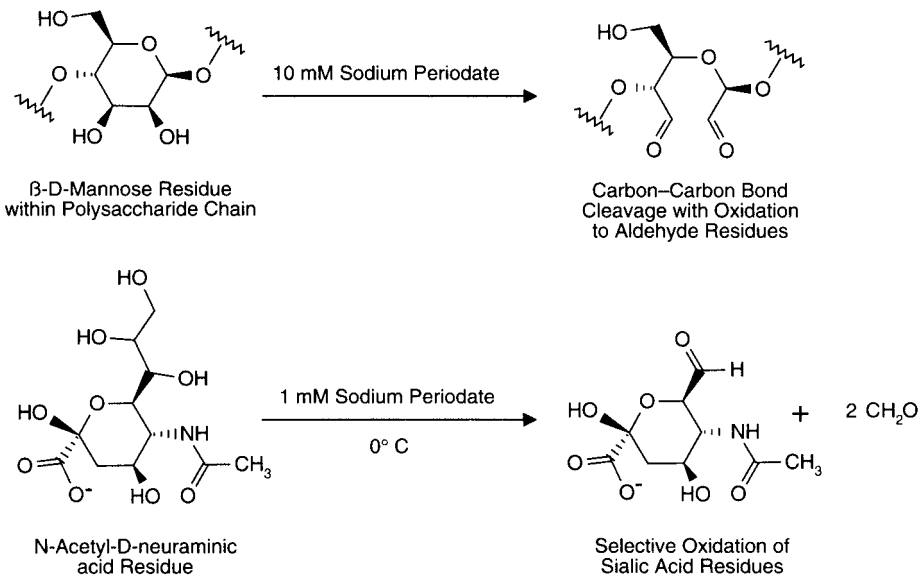


Figure 1.102 The reaction of sodium periodate with sugar residues can produce aldehydes for conjugation reactions.

of periodate result in sugar ring opening and the creation of many aldehydes on each polysaccharide tree.

Using these methods, carbohydrate-containing proteins may be altered to contain aldehydes for conjugation with other proteins or for detection using hydrazide-containing probes (Chapter 9). The aldehydes thus formed then can be coupled to other amine-containing molecules by Schiff base formation and reductive amination (Chapter 2, Section 5.3 and Chapter 3, Section 4). For instance, the enzyme HRP can be activated with periodate for conjugation with antibodies (Nakane and Kawaoi, 1974). Alternatively, such reactive formyl groups may be conjugated to hydrazide-containing molecules to form hydrazone bonds (Chapter 4, Section 8; and Chapter 26, Section 2.1). Cell-surface polysaccharides may be probed with hydrazide-containing reagents for sialic acid groups or total glycoconjugates. Glycoproteins or glycopeptides in solution also may be tagged in this manner. Gangliosides and other glycolipids may be modified with hydrazide reagents as well (Spiegel *et al*., 1982).

Protocol

1. The glycoprotein or diol-containing molecule is dissolved in deionized water or a buffer at physiological pH. Sodium phosphate buffer (0.01–0.1 M), pH 7.0, is an appropriate choice. When oxidizing cell-surface glycoconjugates, use a buffer suitable for cellular stability requirements. Avoid amine-containing buffers such as Tris and glycine, because they may interact with the aldehyde groups as they are formed. For glycoproteins in solution, a concentration range of 1–10 mg/ml will produce acceptable results in this procedure. For sialic acid modification, place the sample in ice to cool to near 0°C.
2. Dissolve sodium periodate (MW 213.91) in water at a concentration of 10 mg/ml (0.046 M). Protect from light. To obtain approximately a 1 mM concentration of sodium periodate in the reaction solution (suitable for oxidizing only sialic acid residues), add 21.8 µl of this stock solution to each ml of the glycoprotein solution to be oxidized. Maintain the solution on ice. For general oxidation of carbohydrates other than just sialic acid, add 218 µl of the stock solution to obtain an approximate final concentration of 10 mM periodate in the reaction. Use room temperature conditions for general carbohydrate oxidation. Wrap the vial containing the reaction solution with aluminum foil to protect from light. The use of an amber vial also is suitable for this purpose.
3. React for 15–30 minutes at room temperature.
4. Quench the reaction by the addition of 0.1 ml of glycerol per ml of reaction solution. Alternatively, the reaction may be stopped by immediate gel filtration on a desalting column. If a dextran-based resin is used for the chromatography, the support itself will react with sodium periodate to quench excess reagent. Alternatively, *N*-acetylmethionine may be added to quench the reaction, because the thioether of the methionine side chain will react with periodate to form sulfoxide or sulfone products (Geoghegan and Stroh, 1992). In addition, sodium sulfite (Na_2SO_3) was used by Stolowitz *et al*. (2001) to quench the periodate oxidation of HRP in solution. To quench the reaction with cellular samples, wash the cells with buffer to remove remaining traces of periodate.

Oxidase Modification of Sugar Residues

Another method of forming aldehyde groups on carbohydrates and glycoproteins involves the use of specific sugar oxidases. These enzymes only affect the monosaccharide they are specific toward,

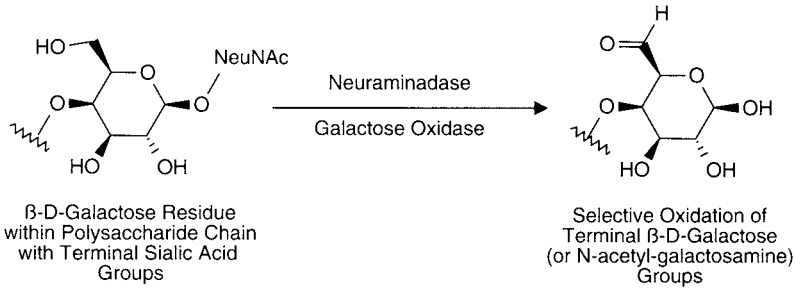


Figure 1.103 Galactose oxidase may be used to transform specifically the C-6 hydroxyl group of galactose into an aldehyde.

leaving other sugar residues within polysaccharides unaffected. Probably the most often used oxidase for this purpose is galactose oxidase, which can form C-6 aldehydes on terminal D-galactose or *N*-acetyl-D-galactose residues (Avigad *et al.*, 1962) (Figure 1.103). When galactose residues are penultimate to sialic acid residues, another enzyme, neuraminidase, must be used to remove the sialic acid sugars and expose galactose as the terminal residue (Wilchek and Bayer, 1987). The specificity of using glycosidases to create aldehyde residues on carbohydrates may be the method's greatest advantage. However, the use of a simple chemical reagent such as sodium periodate still may be the easiest way to create aldehydes on carbohydrates (Section 4.4, this chapter).

The following protocol was used by Wilchek and Bayer (1987) to label cell-surface galactose residues.

Protocol

1. Prepare a 5 percent cell suspension in an appropriate buffer. Avoid amine-containing buffers, as these will interact with aldehydes.
2. Add 0.05 units of *Vibrio cholerae* neuraminidase and 5 units of galactose oxidase per ml of cell suspension.
3. Incubate for 60 minutes at 37°C.
4. Wash cells with PBS to remove excess enzymes.

Modification of Amines with NHS-Aldehydes (SFB and SFPA)

Succinimidyl *p*-formylbenzoate (SFB) and succinimidyl *p*-formylphenoxyacetate (SFPA) are amine-reactive reagents that contain terminal aldehyde residues. Their NHS ester ends react with primary amines in proteins and other molecules at pH 7–9 to yield amide linkages (see Chapter 2, Section 1.4 and Chapter 17, Section 2) (Figure 1.104). The resulting formyl derivatives may be utilized to couple to other amine or hydrazinecontaining molecules (Kraehenbuhl *et al.*, 1974; Galardy *et al.*, 1978). In particular, SFB can be used to produce aldehyde groups on alkaline phosphatase for conjugation with 5′-hydrazide modified DNA for use in hybridization assays (Chapter 27, Section 2.4) (Ghosh *et al.*, 1989). SFB and SFPA are insoluble in water, but may be pre-dissolved in DMF or acetonitrile before adding a small quantity to an aqueous reaction mixture. Both reagents contain aromatic phenyl rings and have absorptivity at wavelengths less than 300 nm. Their structures may contribute a significant degree of

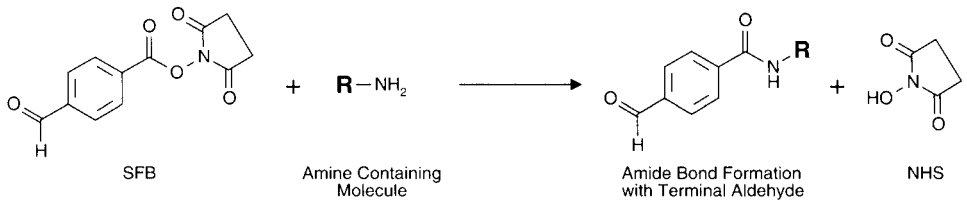
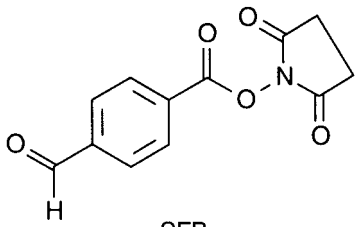
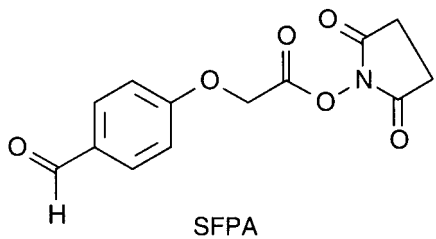


Figure 1.104 SFB reacts with primary amines to form amide bond derivatives containing aldehyde groups.

hydrophobicity to macromolecules being modified, especially if high-density couplings are achieved. For this reason, modified proteins and other soluble molecules may have a tendency to precipitate if modification is done too heavily. The optimal amount of modification may have to be adjusted to maintain solubility in each application.



SFB
Succinimidyl-p-formyl benzoate
MW 247



SFPA
Succinimidyl-p-formylphenoxyacetate
MW 277

Protocol

1. Dissolve a macromolecule containing amine groups at a concentration of 1–10 mg/ml in a buffer having a pH of 7.0–9.0 (i.e., 0.1 M sodium phosphate, pH 7.5). Higher pH conditions will increase the hydrolysis rate of the NHS ester. Avoid amine containing or nucleophilic buffers such as Tris, glycine, or imidazole (see Chapter 2, Section 1.4).
2. Dissolve SFB (Thermo Fisher, Solulink) or SFPA (Molecular Probes) in DMF. The concentration should be such that a small aliquot can be added to the reaction medium to obtain at least a 10-fold molar excess of modifying reagent over the amount of amines

to be modified. Add no more than 100 µl of the modifier/DMF solution to each ml of the macromolecule solution prepared in (1).
3. React for 2 hours at room temperature.
4. Purify the modified macromolecule from excess reagent and reaction by-products by dialysis or gel filtration.

Modification of Amines with Glutaraldehyde

Amino groups on proteins may be reacted with the *bis*-aldehyde compound glutaraldehyde to form activated derivatives able to crosslink with other proteins. The reaction mechanism for this modification proceeds by one of several possible routes. In the first option, one of the aldehyde ends can form a Schiff base linkage with ε-amines or α-amines on proteins to leave the other aldehyde terminal free to conjugate with another molecule. Alternatively, a glutaraldehyde polymer may undergo vinyl addition to create stable secondary amine bonds, leaving the aldehydes exposed for subsequent reductive amination reactions. Finally, a cyclized form of glutaraldehyde also may react with the ε-amines of two neighboring lysine side chains to form a quaternary pyridinium crosslink (Figure 1.105).

Schiff base interactions between aldehydes and amines typically are not stable enough to form irreversible linkages. These bonds may be reduced with sodium cyanoborohydride or a number of other suitable reductants (Chapter 2, Section 5) to form permanent secondary amine bonds. However, proteins crosslinked by glutaraldehyde without reduction nevertheless show stabilities unexplainable by simple Schiff base formation. The stability of such unreduced glutaraldehyde conjugates has been postulated to be due to the vinyl addition mechanism, which doesn't depend on the creation of Schiff bases.

Glutaraldehyde modification readily proceeds at alkaline pH. The higher the pH, the more efficient is Schiff base formation. Using a reductant like sodium cyanoborohydride that does not affect the aldehyde groups, while efficiently transforming Schiff bases into a secondary amines, provides the best possible yields. In many cases, the degree of glutaraldehyde-induced crosslinks is so severe that conjugate precipitation occurs. This is especially well documented in antibody–enzyme conjugation schemes employing this reagent (Chapter 20, Section 1.2).

Glutaraldehyde also can be used to create aldehydes on amine-containing polymers. The use of this reagent in derivatizing chromatography supports and other soluble polymers is well known (Hermanson *et al.*, 1992).

The following protocol may be used as the first stage of a two-step glutaraldehyde conjugation reaction. In this initial reaction, glutaraldehyde modification converts available protein amines into reactive formyl groups. The subsequent addition of a second protein or another amine-containing molecule causes the activated protein to crosslink with the amines and forms a conjugate. Glutaraldehyde also may be used in single-step conjugation procedures where the aldehyde-modified protein is not isolated before addition of a second protein. In single-step conjugation both proteins to be crosslinked are together in solution and glutaraldehyde is added to effect crosslinking (Chapter 20, Section 1.2).

Protocol

1. Dissolve the protein or other amine-containing macromolecule to be modified at a concentration of 1–10 mg/ml in a buffer having a pH from 7 to 10. The higher the pH, the

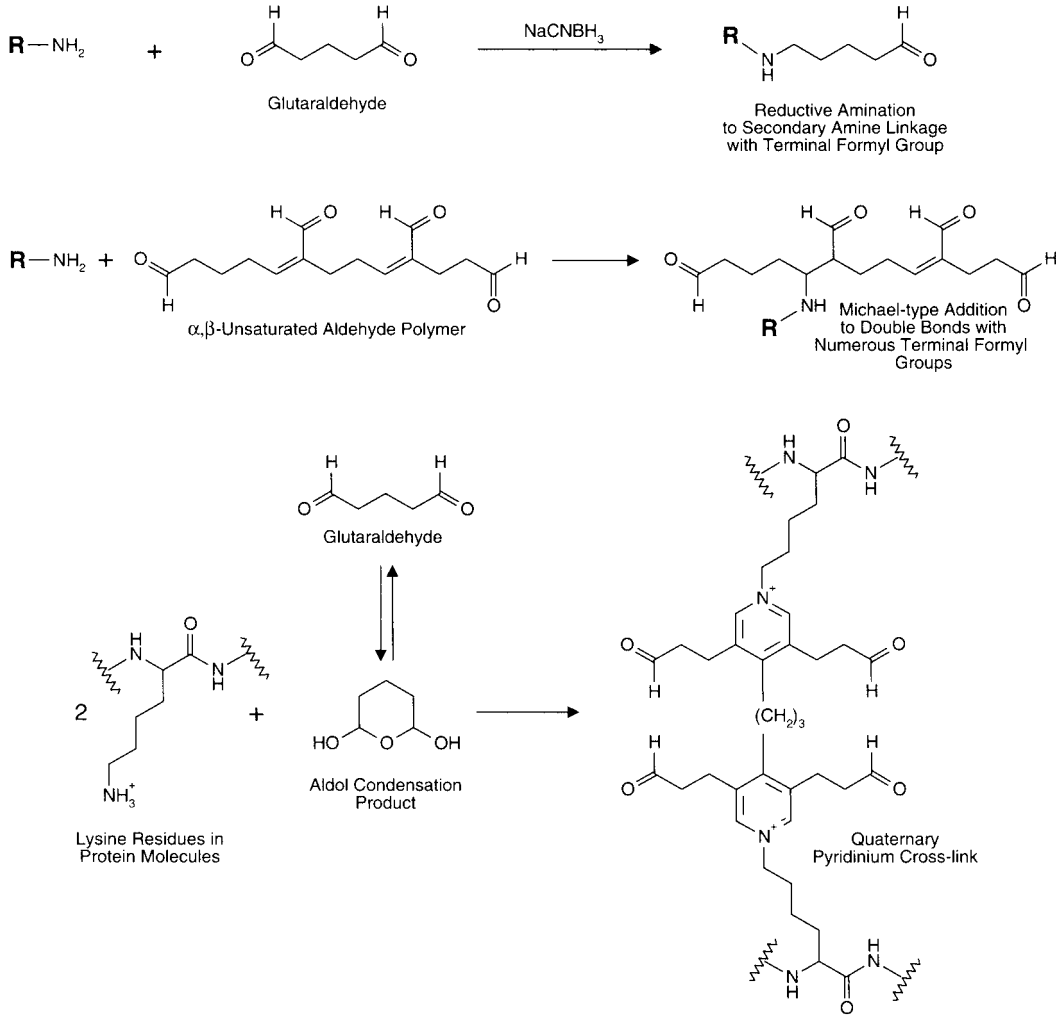


Figure 1.105 Glutaraldehyde can undergo complex reactions with amine groups, resulting in aldehyde-containing derivatives that can be used in conjugation reactions.

more efficiently Schiff base formation will occur. Phosphate, borate, and carbonate buffers at 0.01–0.1 M are acceptable. Avoid amine-containing buffers like Tris and glycine, since they will react with glutaraldehyde.

2. Add a quantity of glutaraldehyde equal to a 10-fold molar excess over the amount of amines to be modified. A typical concentration of glutaraldehyde in the reaction mixture is 1.25 percent. In some cases, trial experiments will have to be done to check for solubility of the resultant modified protein. Scale back the quantity of glutaraldehyde added if precipitation occurs.
3. React for at least 2 hours at 4°C.
4. Quickly isolate the modified protein by gel filtration using a desalting resin.

In some cases, the modified protein may be stored for long periods before conjugation with another amine-containing molecule by immediate freezing and lyophilization. If stability is a problem, however, the modified protein should be conjugated immediately.

Periodate Oxidation of N-Terminal Serine or Threonine Residues

Sodium periodate can be used to form aldehydes on unmodified N-terminal serine or threonine residues in proteins and peptides (Geoghegan and Stroh, 1992). Periodate cleaves carbon–carbon bonds that possess on both of them primary or secondary hydroxyls or amines (i.e., diols or 2-amino alcohol groups). If a primary hydroxyl is present, such as in the case of N-terminal Serine residues, then the reaction liberates formaldehyde and forms an aldehyde group (an α-*N*-glyoxylyl) at the end of the peptide (Figure 1.106). This reaction can be used to direct bioconjugation to a site-specific point on biomolecules, provided that there are no other periodate-oxidizable groups within the protein structure. A synthetic peptide designed to have an N-terminal serine or threonine residue can provide a site of coupling at the end of the chain. This strategy is a viable alternative to the incorporation of a cysteine group for bioconjugation at the end of a peptide.

If other oxidizable groups are present in a protein, such as carbohydrates or sensitive amino acids side chains (Section 1.1, this chapter), then this method should be avoided, because modification can occur at sites other than just the N-terminal. This method has been used successfully to conjugate tags with small peptides, to attach fluorescent probes to enzyme substrates, to effect the conjugation of lactamase to a Fab′ antibody fragment, for the coupling of antibodies to liposomes, and to couple a PEG polymer to the amino terminus of proteins (Geoghegan *et al*., 1993; Gaertner *et al*., 1994; Mikolajczyk *et al*., 1994; Gaertner and Offord, 1996; and Koning *et al*., 1999, respectively).

When using sodium periodate to oxidize an N-terminal serine or threonine residue on a large molecule like a protein, excess oxidant can be removed simply by dialysis or size exclusion chromatography. However, when using periodate to oxidize a low-molecular-weight peptide, it can become problematic to remove excess reactant by size separation methods alone. For this reason, the addition of a reducing agent may be used to scavenge any remaining periodate, so long as the reductant chosen doesn't also reduce the aldehydes that have been formed. Geoghegan and Stroh (1992) used *N*-acetylmethionine for this purpose, because the thioether of the methionine side chain readily can react with periodate to form sulfoxide or sulfone products, but it won't affect the aldehyde groups formed at the end of the peptide chains or interfere with subsequent coupling reactions. A less expensive reagent, sodium sulfite (Na_2SO_3) was used by Stolowitz *et al*. (2001) to quench the periodate oxidation of HRP in solution, and ultimately this may prove to be the best choice for stopping the reaction.

Conjugation of molecules to periodate-oxidized N-terminal serine or threonine residues in peptides is best done using hydrazine or hydrazide reagents to avoid potential cross-reactions with lysine amino groups in the peptide structure. The aldehyde group preferentially reacts with the hydrazino group even in the presence of other amines to form a hydrazone bond (Figure 1.107). After the reaction, the hydrazone may be reduced with sodium cyanoborohydride to stabilize the linkage.

The following protocol is based on the methods of Geoghegan and Stroh (1992) and Stolowitz *et al*. (2001).

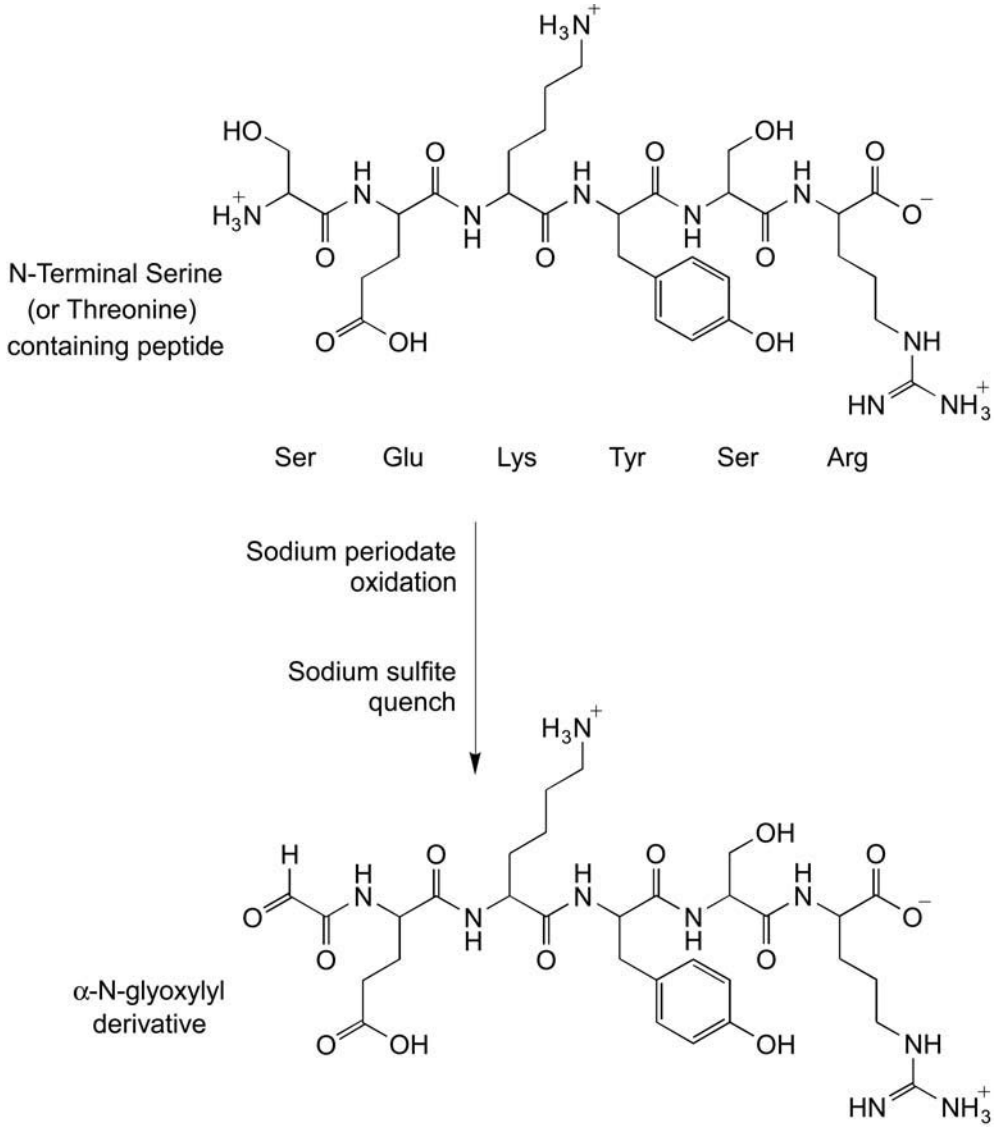


Figure 1.106 An N-terminal serine or threonine residue can be oxidized with sodium periodate to produce an aldehyde group. The reaction can be quenched with sodium sulfite to eliminate excess periodate.

Protocol

1. Dissolve the peptide containing an N-terminal serine or threonine group at a concentration of at least 2 mg/ml in 0.04 M sodium phosphate, pH 7.0. Higher concentrations of peptides or proteins may be used without modification to the rest of the protocol, because the amount of periodate used in the reaction is in sufficient molar excess, even when low-molecular-weight peptides are being oxidized. Peptides that are initially insoluble

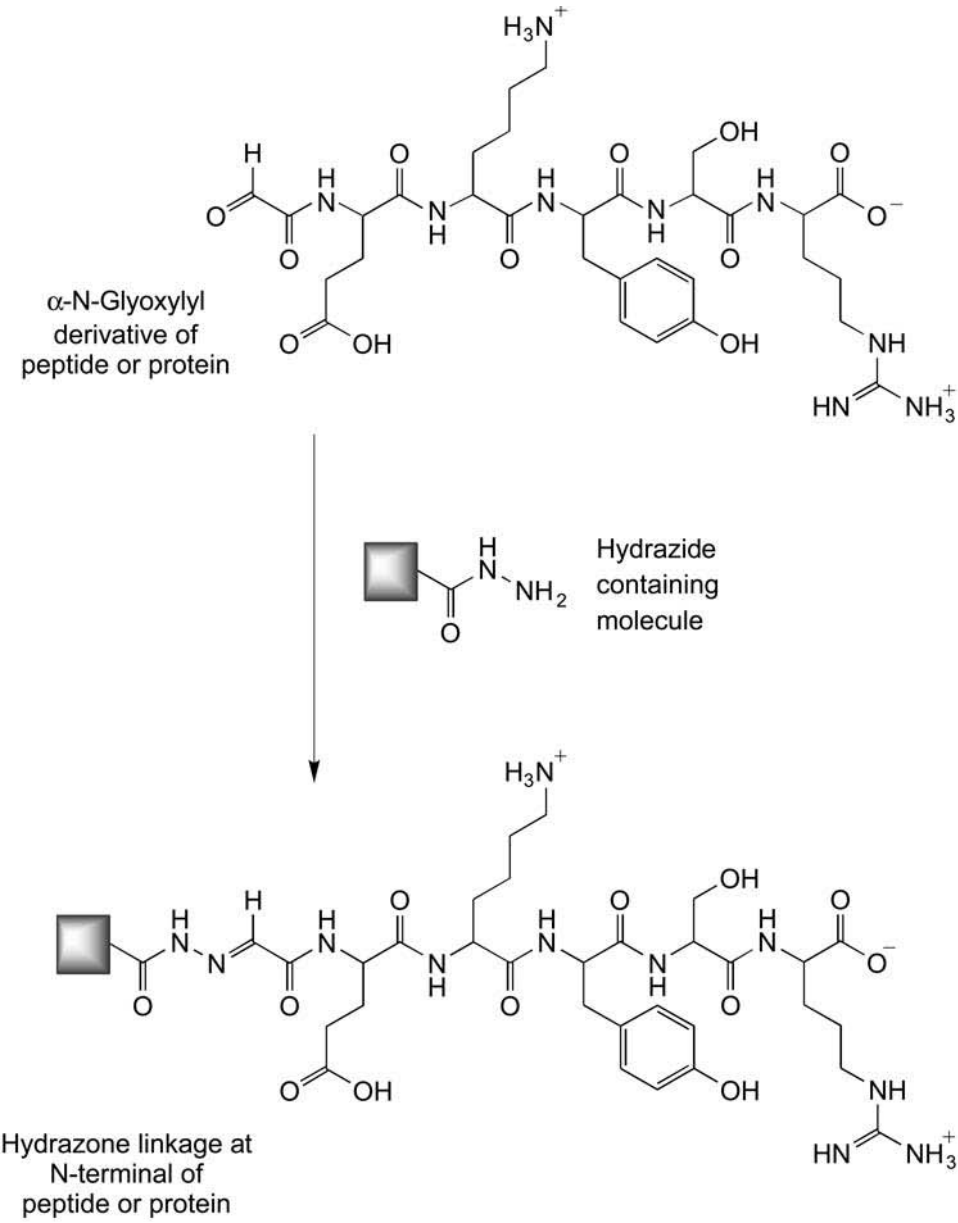


Figure 1.107 The N-terminal aldehyde group on a peptide formed from periodate oxidation of serine or threonine residues can be conjugated with a hydrazide-containing molecule to produce a hydrazone bond.

at physiological pH first may be dissolved at higher concentration in 0.01 percent TFA before adding a small aliquot to the buffer. Adjust the pH back to neutral, if needed.

2. With mixing, add sodium periodate to a final concentration of 2.5 mM. Periodate should be pre-dissolved as a stock solution at a higher concentration in buffer or water, and then

a small aliquot added to the peptide solution to start the reaction. Pre-dissolving the periodate will facilitate immediate dissolution in the final reaction medium without the creation of regions of high concentration, as would occur if solid sodium periodate is added directly to the peptide solution. Protect all solutions containing periodate from exposure to light.

3. React for 3 minutes at room temperature. Longer reactions increase the likelihood of oxidative damage to other amino acids within the peptide structure.
4. Quench the oxidation by the addition to the peptide solution of at least a 4-fold molar excess of *N*-acetylmethionine or sodium sulfite over the concentration of periodate in the reaction mixture. Pre-dissolve the quencher in buffer at a higher concentration prior to adding an aliquot of it to the reaction solution. React for 10 minutes.
5. The oxidized peptide may be reacted with an amine- or hydrazide-containing molecule by reductive amination to conjugate with the newly formed aldehyde at the N-terminal (for protocols see the following section this chapter; Chapter 2, Sections 5.1–5.3; and Chapter 17, Section 2). For conjugation with amine-containing molecules, the peptide must not have any other competing amines (e.g., lysine residues) present or else ring formation or peptide-to-peptide coupling may occur. If lysines are present within the peptide, then the use of a hydrazide (or hydrazine) conjugation process will eliminate interference from lysine amines.

4.5. Introduction of Hydrazine or Hydrazide Functionalities

Hydrazide-containing reagents can be used for probing or conjugation of carbonyl-containing compounds, including macromolecules possessing aldehydes and ketones. Fluorescent or enzymatic probes containing hydrazide functionalities can be used to assay or label carbohydrates, glycoproteins, the polysaccharide portion of cell surfaces, gangliosides, and glycoconjugates on blots (Lotan *et al*., 1975; Hurwitz *et al*., 1980; Spiegel *et al*., 1982; Gershoni *et al*., 1985; Wilchek and Bayer, 1987). Multivalent forms of hydrazide reagents created by modifying enzymes, ferritin, and polymers such as dextran and polypeptides with *bis*-hydrazides can be used to target formyl groups with high avidity and sensitivity (Roffman *et al*., 1980; Kaplan *et al*., 1983).

The creation of hydrazide probes often is based on the derivatization of a detectable molecule with a *bis*-hydrazide compound. Although hydrazine itself (in the form of hydrazine hydrate) can be used in a methanolic solution to modify activated carboxylate molecules forming hydrazides, the availability of the bifunctional hydrazides provides a built-in spacer to accommodate greater steric accessibility.

The following protocols make use of the compounds adipic acid dihydrazide and carbohydrazide to derivatize molecules containing aldehydes, carboxylates, and alkylphosphates. The protocols are applicable for the modification of proteins, including enzymes, soluble polymers such as dextrans and poly-amino acids, and insoluble polymers used as micro-carriers or chromatographic supports.

The addition of hydrazide groups into macromolecules containing aldehydes, carboxylates, or alkylphosphates has the effect of increasing the pI or net charge. In the case of carboxylates or alkylphosphates, blocking these groups with hydrazide compounds eliminates the negative charge contribution of the original functionality and adds a potential positive charge contribution due to the terminal hydrazide. The consequence of raising the pI of a macromolecule can have dramatic effects on the molecule's conformation and activity or on its relative nonspecificity

in assay systems due to the presence of additional positive charge. For instance, the modification of avidin with adipic acid dihydrazide by coupling through the protein's carboxylate groups significantly increases the net charge of an already highly cationic molecule, and therefore increases its overall cross-reactivity in avidin–biotin assays (Chapter 23, Section 5).

Modification of Aldehydes with Bis*-Hydrazide Compounds*

Aldehyde-containing macromolecules will react spontaneously with hydrazide compounds to form hydrazone linkages. The hydrazone bond is a form of Schiff base that is more stable than the Schiff base formed from the interaction of an aldehyde and an amine. The hydrazone, however, may be reduced and further stabilized by the same reductants utilized for reductive amination purposes (Chapter 3, Section 4.8). The addition of sodium cyanoborohydride to a hydrazide–aldehyde reaction drives the equilibrium toward formation of a stable covalent complex. Mallia (1992) found that adipic acid dihydrazide derivatization of periodate-oxidized dextran (containing multiple formyl functionalities) proceeds with much greater yield when sodium cyanoborohydride is present.

The reaction of an excess of adipic acid dihydrazide with aldehyde groups present on glycoproteins or other molecules will result in modified proteins containing alkylhydrazide groups (Figure 1.108). Another *bis*-hydrazide compound, carbohydrazide, also may be employed with similar results, except the spacer afforded through its use is considerably shorter. Target aldehydes may be created on macromolecules according to the protocols described in Section 4.4, this chapter. Thus, glycoproteins and other molecules containing polysaccharides may be periodate oxidized to contain formyl groups and then modified with a *bis*-hydrazide compound to create the hydrazide-activated reagent. Modification of proteins through glycan residues obviates the blocking of negatively charged carboxylates and only adds a limited number of hydrazides at discrete points on the molecule. The enzyme HRP is conveniently modified with hydrazide functionalities using this approach (Chapter 26, Section 2.4).

Protocol

1. Dissolve a macromolecule (such as a protein) containing aldehyde functionalities at a concentration of about 1–10 mg/ml in 100 mM sodium citrate, 150 mM NaCl, pH 6.0. Alternatively, a buffered solution at a pH of about 7–8.5 also will work well in this protocol. Phosphate, carbonate, borate, or similar buffers adjusted to this pH range work well. Avoid amine-containing buffers (i.e., glycine or Tris) or other components containing strong nucleophiles, since these may react with the aldehydes. To modify a molecule to contain aldehyde groups, see Section 4.4, this chapter.
2. Add a quantity of adipic acid dihydrazide or carbohydrazide (Aldrich) to the protein solution to obtain at least a 10-fold molar excess over the amount of aldehyde functionality present. High molar ratios are necessary to avoid protein conjugation during the reaction process. If the concentration of aldehydes is unknown, the addition of 32 mg adipic acid dihydrazide per ml of the protein solution to be modified should work well.
3. React for 2 hours at room temperature. While hydrazone formation does not require the addition of a reductant to create a linkage, including sodium cyanoborohydride in the reaction considerably increases the yield and stability of bonds formed. If the presence

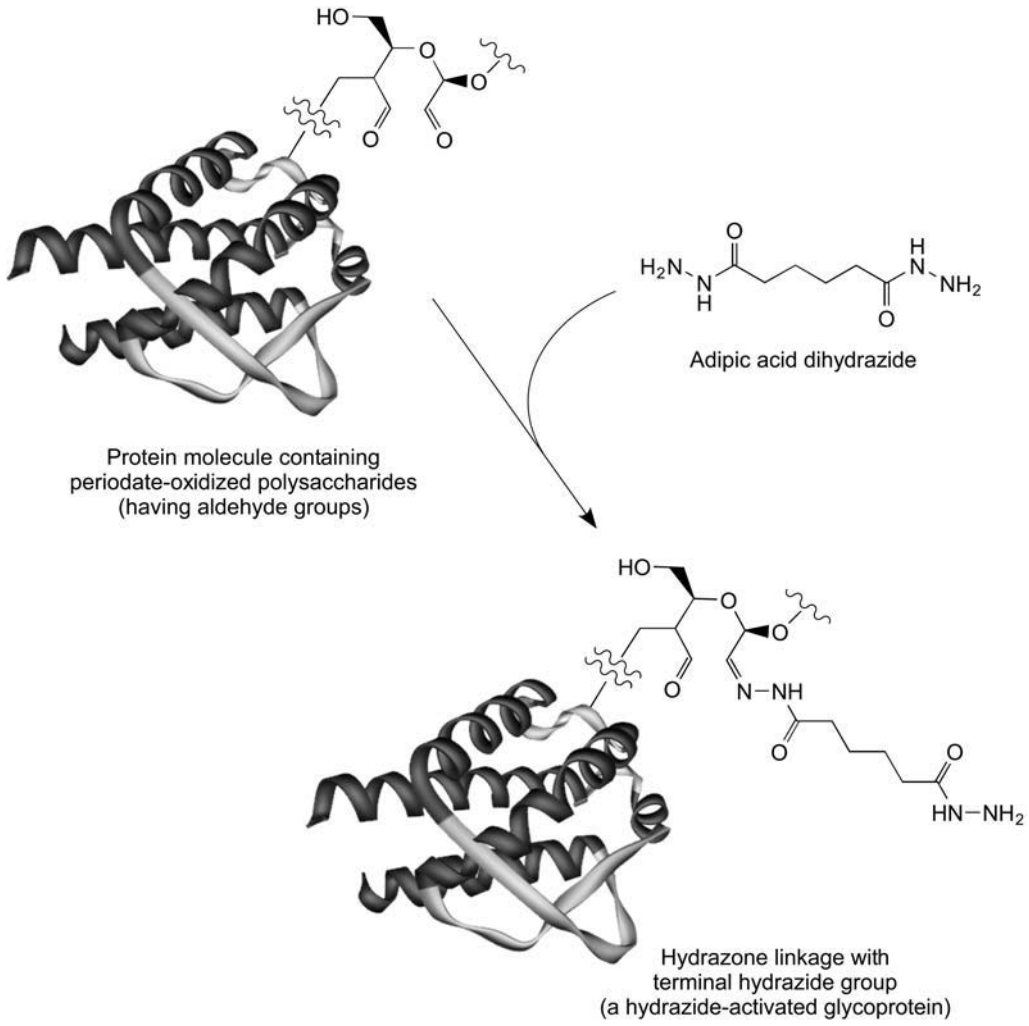


Figure 1.108 Glycoproteins that have been treated with sodium periodate to produce aldehyde groups can be further modified with adipic acid dihydrazide to result in a hydrazide derivative.

of a reducing agent will not cause harm to the macromolecule being modified, the addition of 10 µl of 5 M sodium cyanoborohydride (Sigma) per ml of reaction solution may be done. *Caution*: Cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 M solution is prepared in 1 N NaOH.

4. Purify the modified protein by dialysis or gel filtration using a desalting resin.

Hydrazide-activated proteins are stable to long-term storage at 4°C in the presence of a preservative (0.05 percent sodium azide) or in a frozen or lyophilized state.

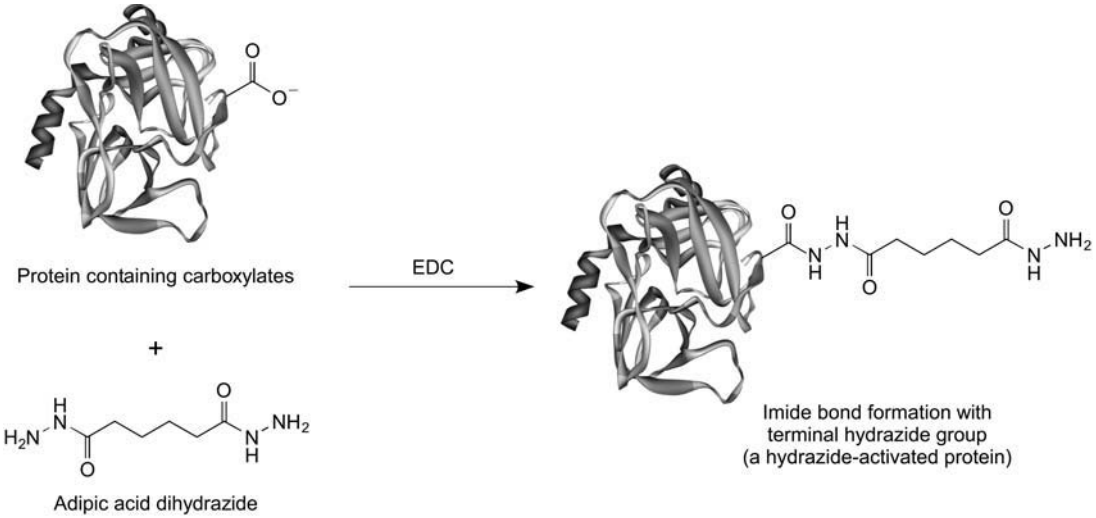


Figure 1.109 Carboxylate groups on proteins may be modified with adipic acid dihydrazide in the presence of a carbodiimide to produce hydrazide derivatives.

Modification of Carboxylates with Bis*-Hydrazide Compounds*

Carboxylic acids may be covalently modified with adipic acid dihydrazide or carbohydrazide to yield stable imide bonds with extending terminal hydrazide groups. Hydrazide functionalities don't spontaneously react with carboxylate groups the way they do with aldehyde groups (Section 4.5, this chapter). In this case, the carboxylic acid first must be activated with another compound that makes it reactive toward nucleophiles. In organic solutions, this may be accomplished by using a water-insoluble carbodiimide (Chapter 3, Section 1.4) or by creating an intermediate active ester, such as an NHS ester (Chapter 2, Section 1.4).

In aqueous solutions, the easiest method for forming this type of bond is to use the water-soluble carbodiimide EDC (Chapter 3, Section 1.1). For proteins and other water-soluble macromolecules, EDC reacts with their available carboxylate groups to form an intermediate, highly reactive, *o*-acylisourea. This active ester species may further react with nucleophiles such as a hydrazide to yield a stable imide product (Figure 1.109).

Most proteins contain an abundance of carboxylic acid groups from C-terminal functionalities and aspartic and glutamic acid side chains. These groups are readily modified with *bis*-hydrazide compounds to yield useful hydrazide-activated derivatives. Both carbohydrazide and adipic acid dihydrazide have been employed in forming these modifications using the carbodiimide reaction (Wilchek and Bayer, 1987).

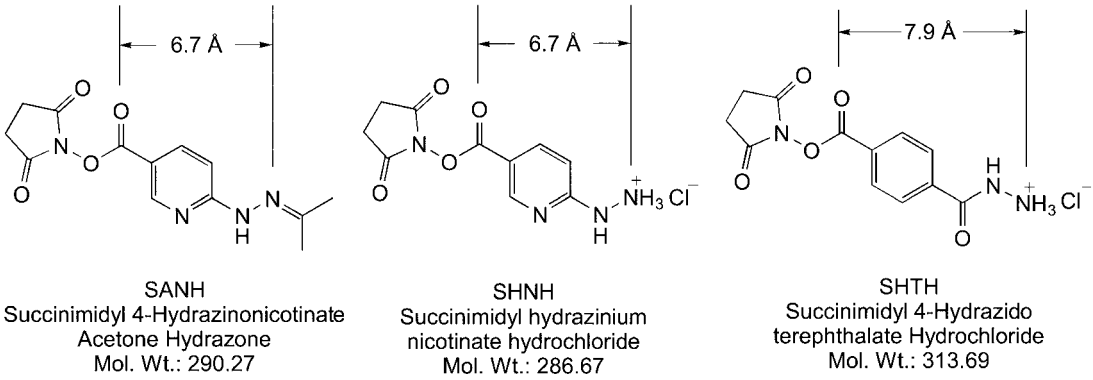
Protocol

1. Dissolve 32 mg of adipic acid dihydrazide per ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. Dissolve 5 mg of the protein or other macromolecule to be modified per ml of the above solution.

3. Add 16 mg EDC and react at room temperature for 2–4 hours.
4. Purify the modified protein by dialysis or gel filtration using a desalting resin.

Modification of Amines with SANH, SHNH, or SHTH

The introduction of hydrazine or hydrazide functional groups may be done using the bifunctional crosslinkers that are a part of the hydrazine-aldehyde chemoselective ligation reagents, which are described in Chapter 17, Section 2 (Wong, 1991; Hartmann *et al.*, 2002; Zhong *et al.*, 2003; Kozlov *et al.*, 2004). These are amine-reactive compounds that all have NHS esters on one end, and which form amide bonds when coupled to primary amines, such as on the side chain of lysine. SANH (succinimidyl 4-hydrazinonicotinate acetone hydrazone) and SHNH (succinimidyl hydraziniumnicotinate hydrochloride) both are hydrazine derivatives of nicotinic acid, while SHTH (succinimidyl 4-hydrazidoterephthalate hydrochloride) is a hydrazide derivative of terephthalate. Hydrazines and hydrazides react with carbonyl compounds, such as aldehydes and ketones, to form hydrazone bonds. The hydrazone linkage formed between a hydrazine and an aldehyde is much more stable than that formed between a hydrazide and an aldehyde. Thus, hydrazine–aldehyde bonds typically don't require further reduction to stabilize the linkage, as is often the case with hydrazide–aldehyde linkages. For both hydrazide and hydrazine groups, however, the bond that is formed with ketones is typically weaker than the hydrazone formed with aldehydes.



SANH
Succinimidyl 4-Hydrazinonicotinate
Acetone Hydrazone
Mol. Wt.: 290.27

SHNH
Succinimidyl hydrazinium
nicotinate hydrochloride
Mol. Wt.: 286.67

SHTH
Succinimidyl 4-Hydrazido
terephthalate Hydrochloride
Mol. Wt.: 313.69

The reaction of SANH, SNHN, or SHTH with an amine-containing molecule proceeds at physiological pH or at slightly basic pH conditions to form an amide bond (Figure 1.110). Reported reaction conditions used the following buffers to modify proteins, amine-modified DNA, or amine-modified surfaces: (a) 0.1 M sodium borate buffer, pH 8.1–8.4 or (b) 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.4. Whereas NHS esters are reactive throughout this range, the use of pH buffers closer to neutrality will provide optimal yields while reducing the amount of competing hydrolysis as compared to higher pH conditions. Avoid the use of amine or thiol components in the reaction medium, as these will react with the NHS ester (e.g., avoid Tris buffer, glycine, DTT, or imidazole).

The following protocol describes a general method for modifying amines with SANH, SNHN, or SHTH and assaying for the incorporation of hydrazino groups in the final derivative.

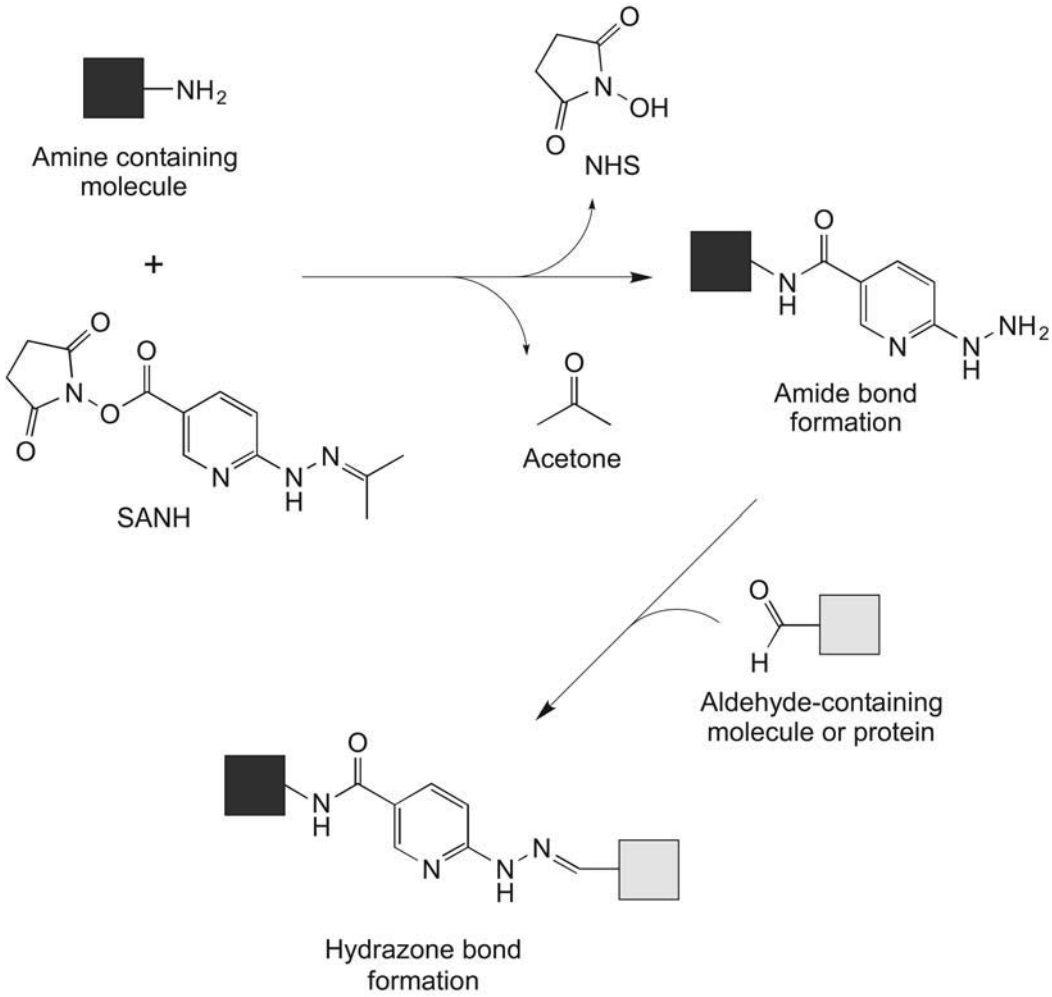


Figure 1.110 The reaction of SANH with an amine-containing molecule results in an amide bond derivative that terminates in a protected hydrazine group. Reaction with an aldehyde-containing molecule results in release of the acetone-protecting group and formation of a stable hydrazone bond.

Protocol

A. Modification of amine groups on proteins with SANH, SHNH, or SHTH

1. Dissolve SANH, SHNH, or SHTH in DMF to prepare a stock solution at a concentration of 2.0–4.0 mg in 100–200 µl. Use highly pure and dry solvent (H_2O content < 0.1 percent or treat with molecular sieves) to prevent hydrolysis of the NHS ester.
2. Dissolve the protein to be modified in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.4, at a concentration of at least 1–10 mg/ml.
3. With mixing, add an aliquot of the crosslinker solution to the protein solution such that the desired molar excess is attained of reagent over the protein present in the reaction.

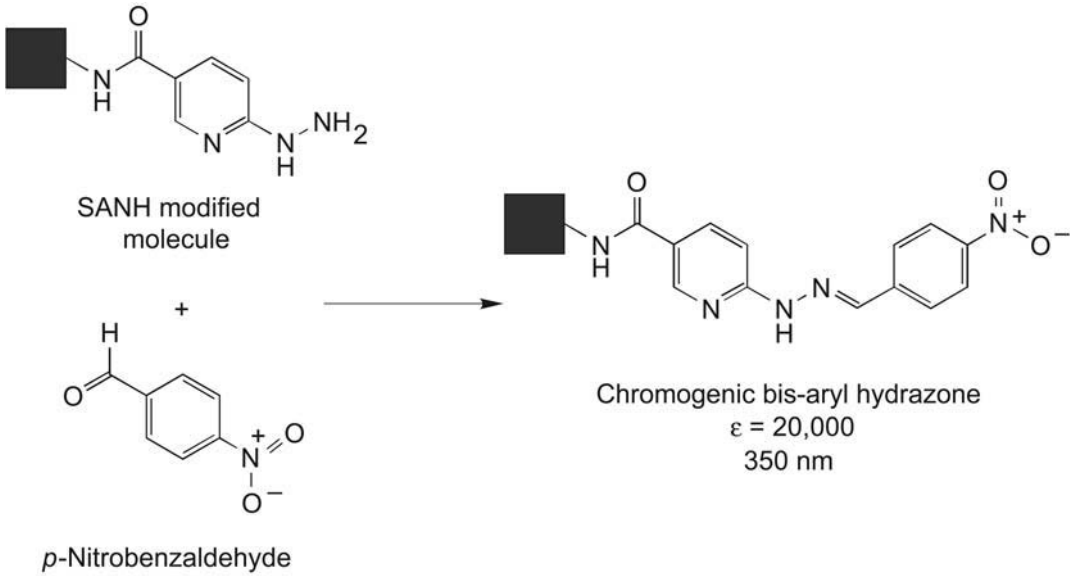


Figure 1.111 An SANH-modified molecule can be detected and measured by reaction with *p*-nitrobenzaldehyde, which forms a chromogenic derivative with a characteristic absorbance at 350 nm.

For most applications, molar ratios in the range of 5:1 to 20:1 will work best to generate a number of hydrazine groups on the protein. Maintain the final percentage of DMF in the reaction mixture at less than 10 percent to avoid precipitation of protein.

4. React at room temperature for at least 30–60 minutes or 2–3 hours at 4°C.
5. Purify the modified protein away from excess reagent and reaction by-products by gel filtration using a desalting column or dialysis.
6. Calculate the protein concentration in the final preparation using its absorbance at 280 nm or a colorimetric method, such as the Coomassie assay. (*Note*: The presence of hydrazine or hydrazide groups on the protein will interfere with the BCA assay for total protein concentration.)

B. Measurement of hydrazine modification level

The number of hydrazine groups per protein molecule can be determined by reacting a small portion of the hydrazine-modified protein with *p*-nitrobenzaldehyde, which forms a chromogenic product upon formation of the hydrazone derivative (Figure 1.111).

1. Prepare a 0.5 mM *p*-nitrobenzaldehyde buffer by initially dissolving the compound at a higher concentration in organic solvent (e.g., methanol, DMF, or DMSO) and then adding the appropriate aliquot to 100 mM acetate, pH 5.0 to result in the final concentration.
2. Add an aliquot of the hydrazine-modified protein solution to the *p*-nitrobenzaldehyde solution and incubate at 37°C for 1 hour or at room temperature for 2 hours. To assure accuracy, determine the linear response range of the test by adding a series of different concentrations of the hydrazine-modified protein solution to the *p*-nitrobenzaldehyde buffer. This is done by preparing a set of serial dilutions of the protein solution and

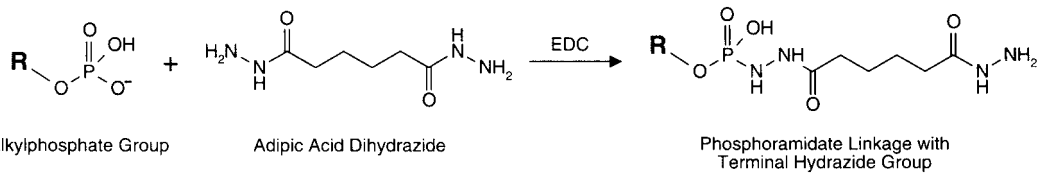


Figure 1.112 Phosphate groups can be modified with adipic acid dihydrazide in the presence of a carbodiimide to produce hydrazide derivatives. This is a common modification route for the 5′-phosphate group of oligonucleotides.

adding an equal volume of each dilution to an aliquot of the *p*-nitrobenzaldehyde solution in separate tubes. Determine the absorbance of each solution at 380 nm versus a blank prepared by the addition of an equal aliquot of buffer alone.

3. Calculate the substitution level of hydrazine groups into the protein by determining the hydrazine concentration using the molar extinction coefficient of the resultant *p*-nitrobenzyl derivative (22,000 $M^{-1}cm^{-1}$) and dividing this value by the protein concentration (moles/liter). Any sample falling within the linear response range of the test can be used for this calculation.

Modification of Alkylphosphates with Bis*-Hydrazide Compounds*

Alkylphosphate groups such as those present at the 5′-end of RNA and DNA molecules may be specifically modified with *bis*-hydrazide compounds. Mediated by the addition of the water-soluble carbodiimide EDC and imidazole, adipic acid dihydrazide or carbohydrazide will react with the phosphate group in a two-step process to form phosphoramidate bonds with short linker arms containing terminal hydrazides (Figure 1.112) (Ghosh *et al.*, 1989). In the first stage, EDC activates the phosphate group forming a short-lived, but highly reactive phosphodiester species, which in turn reacts with a molecule of imidazole to form a longer-lived, active phosphorimidazolide. The second stage involves addition and attack of the hydrazide nucleophile, releasing imidazole and forming the phosphoramidate bond. In a modification of the two-stage reaction, Zanocco (1993) developed a single-pot reaction in which the alkylphosphate molecule is reacted in the presence of EDC, imidazole, and the bis-hydrazide compound. The modification reaction proceeds rapidly at room temperature.

Protocol

1. Weigh out 1.25 mg of the carbodiimide EDC hydrochloride (Thermo Fisher); into a microfuge tube.
2. Add to the tube 7.5 µl of RNA or DNA containing a 5′-phosphate group. The concentration of the oligonucleotide should be 7.5–15 nmol or total of about 57–115.5 µg. Also, immediately add 5 µl of 0.25 M adipic acid dihydrazide or carbohydrazide dissolved in 0.1 M imidazole, pH 6.0. Because EDC is labile in aqueous solutions, the addition of the oligo and *bis*-hydrazide/imidazole solutions should occur quickly.
3. Mix by vortexing, then place the tube in a microcentrifuge and spin for 5 minutes at maximal rpm.

4. Add an additional 20 µl of 0.1 M imidazole, pH 6.0. Mix and react for at least 2 hours at room temperature. The additional buffer prevents pH drift during the carbodiimide reaction.
5. Purify the hydrazide-labeled oligo by gel filtration on a desalting resin using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. The hydrazide-containing probe now may be used to conjugate with a molecule containing an aldehyde-reactive group.

4.6. Introduction of Saccharide or Glycan Groups

The modification of proteins with sugar groups occurs *in vivo* through both enzymatic and non-enzymatic processes. Approximately 1 percent of proteins encoded in the genomes of mammals are enzymes that are involved with carbohydrate production or modification. Many of these enzymes digest carbohydrate in foods to provide energy for cellular metabolism, but others are involved with the controlled modification of proteins or other biomolecules to create complex polysaccharide structures. This process results in carbohydrates, called glycans, covalently attached to proteins at discrete locations on only certain amino acid residues within a polypeptide sequence (Section 2, this chapter). The presence of carbohydrate modifications on proteins has a pronounced effect on biological activity *in vivo*.

Non-enzymatic modification of proteins with saccharides also occurs *in vivo* through uncontrolled glycation of lysine amines with the reducing end of sugars, especially glucose. This reaction results in the formation of an initial Schiff base with a subsequent rearrangement to form a stable ketoamine derivative. The non-enzymatic glycation reaction has been studied extensively as a result of it being a major factor in the development of the complications associated with diabetes (for review, see Singh *et al.*, 2001).

In vitro modification of protein can be done synthetically to add specific sugars or complex carbohydrates to proteins for further bioconjugation or for subsequent study of the glycan-derivative *in vivo*. Investigations of the effect of these synthetic carbohydrate–protein conjugates (neoglycoproteins) on the immune response date back many decades with the diazonium-mediated coupling of aminophenol glucosides to study type-II and type-III pneumonia polysaccharides (Goebel *et al.*, 1932). More recently, conjugation of carbohydrates to protein carriers has been done to illicit a specific immune response to glyco-antigens of infectious diseases or tumor cells (Toyokuni and Singhal, 1995; Koganty *et al.*, 1996; Ragupathi *et al.*, 1997; Pozsgay, 1998; Mawas *et al.*, 2002; Karsten *et al.*, 2004). Synthetic peptide–glycan conjugates also have been prepared by conjugation of carbohydrates to peptide sequences that can be presented by MHC (major histocompatibility complex) molecules to enhance the immune response against the carbohydrate component (Kihlberg and Magnusson, 1996). For an excellent review of glycoconjugation, see Davis (1999).

Sugar residues also can be used to modify a protein, molecule, or surface for subsequent use in a bioconjugation procedure or to increase the hydrophilicity of the modified molecule. For bioconjugation purposes, a sugar group can be added to facilitate the covalent conjugation of another molecule. Since many saccharides contain diols that can be oxidized by periodate to create aldehydes, certain sugars can be used after glycol oxidation to couple with amine-containing molecules by reductive amination. For instance, the amine group on the monosaccharide glucosamine can be coupled to an amine-reactive surface or to a protein through its carboxylate groups using EDC (Chapter 3, Section 1.1). The glucosamine-modified surface or molecule

subsequently can be oxidized by sodium periodate to create aldehydes (Section 2 and 4.4, this chapter). This reactive intermediate finally can be conjugated to another amine-containing molecule to create the final conjugate through reductive amination (Chapter 2, Section 5).

Periodate oxidation of carbohydrates should be avoided, however, if generating an immune response against the saccharide component is desired or antibody recognition needs to be retained against the carbohydrate. Woodward *et al*. (1985) demonstrated that periodate oxidation of glycans effectively destroys antibody binding if the specificity of the antibody truly is toward the carbohydrate. The assay of antibody binding to a carbohydrate with and without periodate oxidation often is used to demonstrate antibody specificity. A specific antibody will fail to bind to periodate-oxidized carbohydrate, but will bind to the non-oxidized glycan.

The modification of molecules with saccharides also has the effect of increasing the hydrophilicity of the resultant complex due to the presence of multiple hydroxyl groups. Native glycan modification of proteins functions in much the same manner, because the carbohydrate

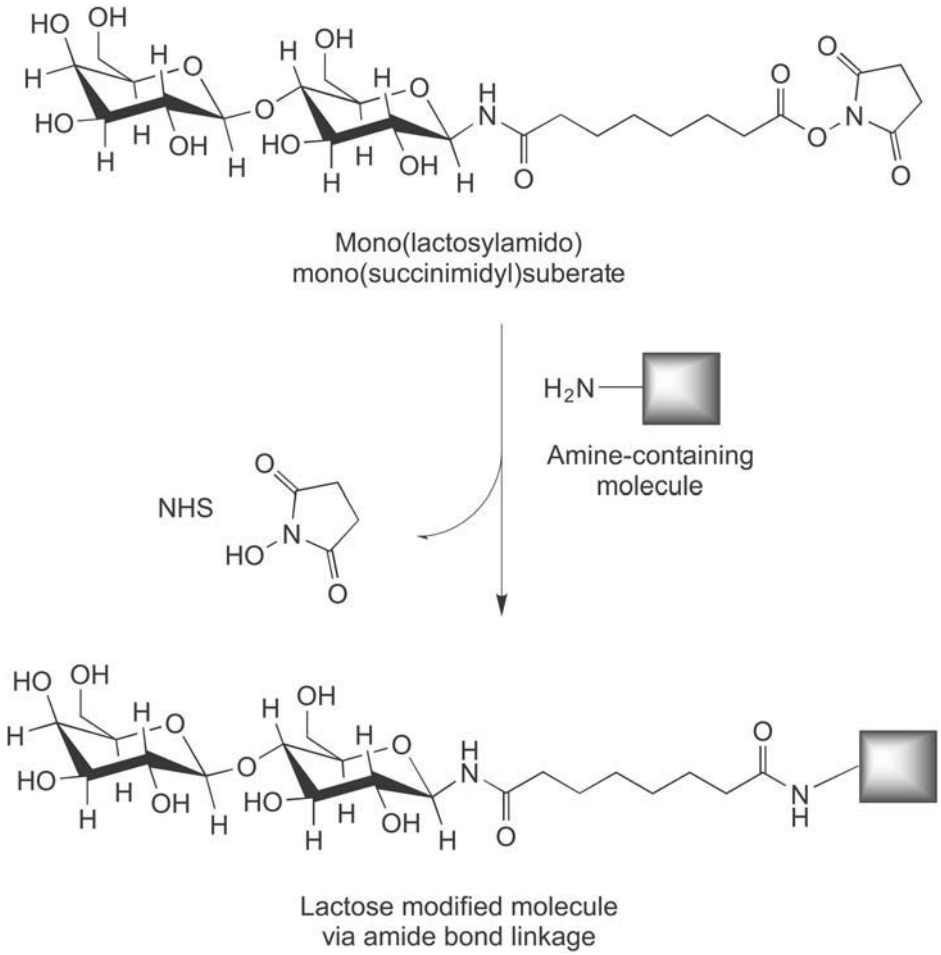


Figure 1.113 The NHS ester–suberate derivative of lactose can be used to add lactose groups to amine-containing molecules. The reaction results in the formation of amide bonds containing terminal lactose groups.

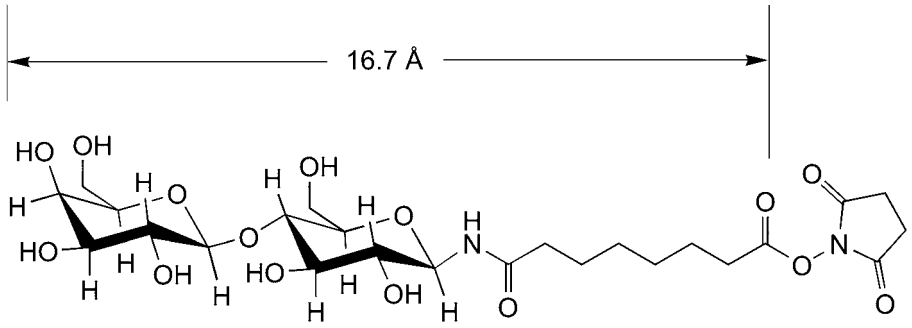
"tree" becomes fully hydrated through the ability of the hydroxyls to hydrogen bond with the surrounding water molecules. In addition, post-translational modification of proteins helps polypeptides fold properly by assuring that certain regions are maintained near the solvent surface. Chemical modification with saccharides thus can be done to increase the hydrophilicity and solubility of proteins or other molecules in aqueous solution.

Some reports even indicate that the conjugation of proteins or peptides with carbohydrates can increase dramatically their activity compared to that of the native state (Susaki *et al.*, 1998). Carbohydrates also can provide a protective effect on modified peptides toward proteolytic digestion (Rudd *et al.*, 1994) or mask recognition of a peptide by the immune system (Harding *et al.*, 1993). The creation of neoglycoproteins thus can affect the activity of peptides and proteins, which are not normally glycosylated *in vivo*.

The following sections describe several examples of saccharide modification for the purpose of bioconjugation, the study of glycan function, to prepare immunogens, or to increase the water solubility of a modified molecule.

Modification of Amines with Mono(lactosylamido) mono(succinimidyl)suberate

The amine-reactive compound mono(lactosylamido) mono(succinimidyl)suberate contains a lactose group at the end of a suberate bridge, which terminates at the other end in an NHS ester. Vetter *et al.* (1995) prepared this reagent by reacting the corresponding glycosylamine derivative with disuccinimidyl suberate (DSS). The starting lactosylamine derivative was prepared via a 5-day reaction of the reducing end of lactose with aqueous ammonia solution in sodium carbonate. Other similar 1-amino, 1-deoxy sugar derivatives may be synthesized in a like manner for subsequent bioconjugation. The final product after reaction with DSS, the mono-NHS ester derivative of lactose containing an 8-carbon suberate spacer, can be used to modify proteins or other amine-containing molecules with a lactose group. The NHS ester spontaneously reacts with an amine at neutral or slightly basic pH values to form an amide bond (Figure 1.113). The presence of the lactose disaccharide provides a hydrophilic modifying group that increases the water solubility of the resultant conjugate. Other glycosylamine derivatives may be prepared using a similar strategy to modify biomolecules or surfaces with specific saccharide compounds.



Mono(lactosylamido)
mono(succinimidyl)suberate
MW 594.56

The following protocol describes the modification of a protein with mono(lactosylamido) mono(succinimidyl)suberate. The reagent is available from Thermo Fisher. The use of this reagent to couple to amine-containing surfaces, such as polystyrene beads, also has been done using similar reaction conditions (Vetter *et al.*, 1995).

Protocol

1. Dissolve mono(lactosylamido) mono(succinimidyl)suberate in dry DMF to prepare a concentrated solution from which an aliquot may be taken and added to a final aqueous reaction medium. The compound is extremely soluble in DMF, and solutions of 100 mg/ml may be prepared. The use of dry solvent is essential to prevent hydrolysis of the NHS ester. However, make only enough of this stock solution so that a small amount added to the protein reaction will provide the appropriate molar excess desired for the modification reaction.
2. Prepare the protein to be modified in a non-amine-containing buffer at a slightly basic pH (i.e., avoid Tris or imidazole). The use of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 works well for NHS ester reactions. The concentration of the protein in the reaction buffer may vary from μg/ml to mg/ml, but highly dilute solutions will result in less efficient modification yields. A protein concentration from 1 to 10 mg/ml works well in this reaction.
3. With mixing, add a quantity of the mono(lactosylamido) mono(succinimidyl)suberate in dry DMF to the protein solution to result in a 10–20 fold molar excess of reagent over the amount of protein present. Depending on the desired application for the lactosyl-modified protein, several different molar ratios of reactant-to-protein may have to be tried to optimize the resulting modification level.
4. React for 30–60 minutes at room temperature with gentle mixing.
5. Purify the modified protein away from reactants and reaction by-products using dialysis or size exclusion chromatography.

Modification of Amine or Hydrazide Molecules by Carbohydrates and Glycans

The modification of proteins, surfaces, or other molecules with reducing sugars through a reductive amination process still is perhaps the most common method for glycosyl addition. A saccharide or glycan molecule containing a reducing end typically has a masked carbonyl group (i.e., an aldehyde or ketone) that can be reacted with an amine or hydrazide in the presence of a reducing agent (such as sodium cyanoborohydride or borane compounds) to form a secondary amine or a reduced hydrazone bond (Figure 1.114). However, since most reducing ends of sugars or glycans exist mainly in an acetal (or ketal) ring form, while only the open form with the exposed aldehyde can participate in a reductive amination reaction, the rate of modification by this process can be slow and the yield low.

To help overcome the predominance of the cyclic acetal form, which inhibits the desired amination reaction, functional groups other than amines (having greater reactivity) have been used, along with elevated temperatures and high concentrations of reducing agent, to form more efficiently the initial Schiff base and thus drive the coupling reaction to completion. In particular, hydrazide or hydrazine groups have been used successfully to modify reducing

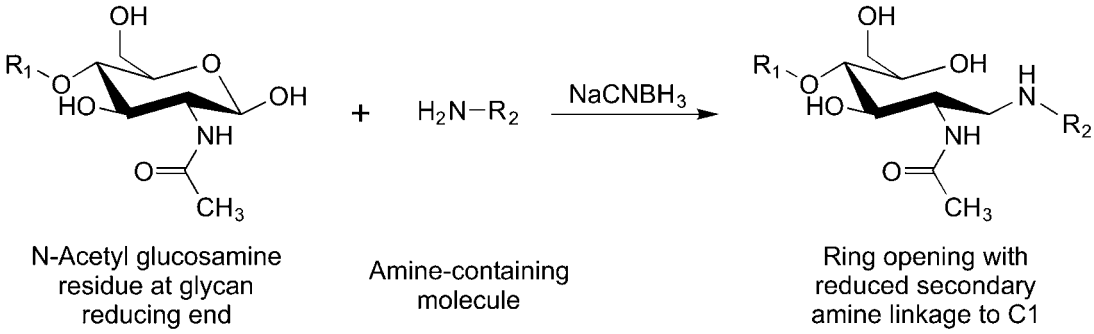


Figure 1.114 The reaction of a reducing sugar with an amine-containing compound in the presence of sodium cyanoborohydride results in ring opening with the formation of a secondary amine derivative.

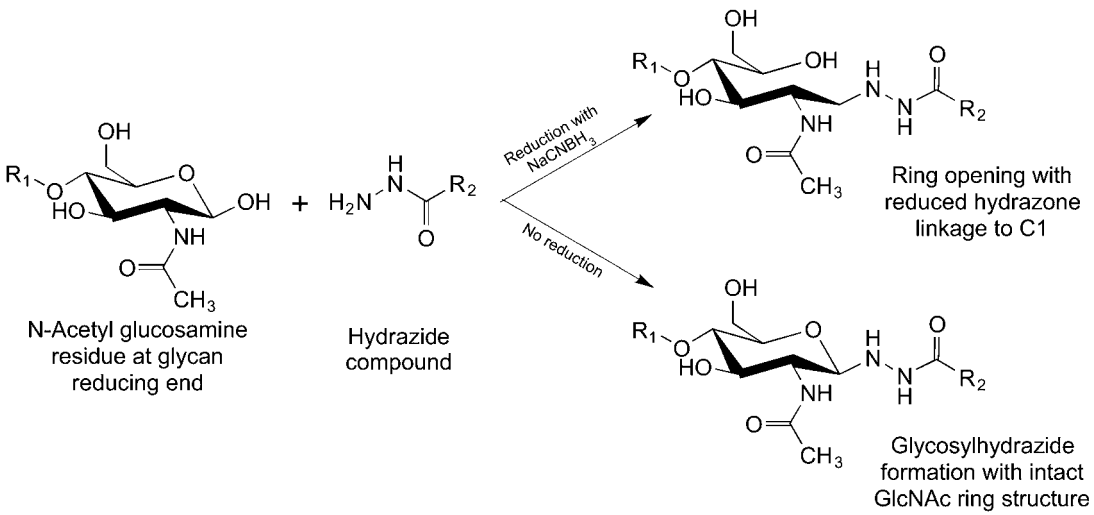


Figure 1.115 The reaction of a reducing sugar with a hydrazide-containing molecule can proceed by one of two routes depending on whether a reducing agent is present. If the reaction is done in the presence of sodium cyanoborohydride, then ring opening will occur followed by the formation of a reduced hydrazone linkage. If no reducing agent is present, then the reaction gives a glycosylhydrazide derivative with retention of the ring structure of the sugar group.

glycans and saccharides with or without a reducing agent present (Rothenberg *et al.*, 1993; Toomre and Varki, 1994; Leteux *et al.*, 1998; Srikrishna *et al.*, 2001) (Figure 1.115). Whereas reactions done with primary amines at room temperature may take days to reach acceptable coupling yields, the modification of hydrazino groups with the reducing end of a sugar can be done in hours at 60–80°C. Also, the modification of glycans with hydrazines can be done without the addition of cyanoborohydride, because the resultant hydrazone linkage is more stable compared to the Schiff base formed between an aldehyde and an amine. Although when coupling hydrazino compounds to the reducing end of a glycan, the addition of a reductant often

is done to drive the reaction to completion and further stabilize the hydrazone, reduction of the terminal saccharide can cause changes in the binding potential of some lectins that recognize the core sugar structure (Leteux *et al.*, 1998).

The following protocol may be used to conjugate the available reducing end of a saccharide or glycan with an amine, hydrazide, or hydrazine group. These functional groups may be present on a variety of molecules, such as biotin groups for labeling or molecules having fluorescent properties, which allows detection of the glycan derivative. For specific modification details, see the methods of Rothenberg *et al.* (1993); Toomre and Varki (1994); Leteux *et al.* (1998); and Srikrishna *et al.* (2001). The following method is based on the optimized conditions as determined by Bigge *et al.* (1995). The use of organic solvents should be done in a fume hood.

Protocol

1. Dissolve a carbohydrate, saccharide, or glycan sample having a free reducing end in 0.1 M sodium acetate, pH 5.0 (*Note*: Glycans may be released from glycoconjugates by hydrazinolysis using pure hydrazine or by endoglycosidase treatment with PNGase F). Alternative coupling conditions that can be used for the modification reaction include 30 percent glacial acetic acid in DMSO (v/v) or acetic acid/pyridine (1:2, v/v). The use of DMSO or pyridine often facilitates solubilization of a greater range of carbohydrates or glycans than aqueous buffers. The presence of acetic acid has been found to accelerate the reductive amination reaction when the organic solvent conditions are used (Bigge *et al.*, 1995). The concentration of the carbohydrate should be 5–100 μM for glycans. For modification of other more abundant carbohydrates, higher concentrations can be used if required. For glycan modification of biomolecules that are not compatible with organic solvents, such as proteins, the glycan initially may be solubilized in DMSO and then an aliquot added to the aqueous reaction buffer.
2. Add to the glycan solution the molecule to be labeled containing an available amine, hydrazine, or hydrazide group. For small molecule derivatization, the final concentration of the nucleophile in the glycan solution should be about 0.3 M to result in maximal efficiency of labeling. For protein modification, an aqueous reaction buffer should be used, and the protein should be as concentrated as possible.
3. Add to the reaction mixture a quantity of reducing agent (e.g., sodium cyanoborohydride or borane dimethylamine (BDA)) to give a final concentration of 1.0 M.
4. When using nonaqueous reaction conditions, incubate for 1–2 hours at 60–80°C. For reactions in an aqueous environment with temperature-sensitive molecules, the reaction may be done at room temperature or 37°C. In this case, the reaction time should be extended to at least 24 hours. Longer reaction times are not unusual when modifying carbohydrates by reductive amination at ambient temperature. For instance, the coupling of heparin through its reducing end to a solid phase containing a hydrazide group takes up to 72 hours at room temperature to obtain maximal yield.
5. Purify the modified glycan from reactants and reaction by-products by dialysis, gel filtration, or ion-exchange chromatography, depending on the size and type of the molecule being modified by the carbohydrate. For instance, Rothenberg *et al.* (1993), fractionated glycans modified with a biotin–diaminopyridine derivative from excess biotin compound by size exclusion chromatography on a 1.5 × 48 cm Toyopearl HW40S column equilibrated with 50 percent acetonitrile/10 mM sodium acetate.

Labeling Glycans with Fluorescent 2-Aminopyridine, 2-Amino Benzamide, or Anthranilic Acid

The ability to label glycans released from glycoproteins and other glycoconjugates is important for tracking complex carbohydrates through purification or analysis, such as HPLC, electrophoresis, and mass spec. Glycan molecules typically don't have spectrally detectable groups and therefore benefit by being labeled with a detectable component for easy assay and detection. Small, amine-containing fluorescent compounds have been found to be particularly useful in this regard. The compounds 2-aminopyridine, benzamide, and anthranilic acid (2-aminobenzoic acid) have been used to label the reducing end of glycans and other carbohydrates.

Glycosylated proteins and other glycoconjugates may be treated to release the modifying carbohydrate component, making available the reducing end for conjugation. *N*-linked glycans may be released from glycoconjugates by PNGase F, PNGase A or released by anhydrous hydrazine and regenerated to reducing oligosaccharides, thus yielding free reducing ends for bioconjugation. In addition, the enzymes Endo H and Endo F release *N*-linked glycans by cleaving the GlcNAc(β1–4)GlcNAc core, thereby resulting in one less GlcNAc moiety at the reducing end. *o*-Linked glycans may be released using *o*-glycanase treatment, or by mild hydrazinolysis followed by regeneration to the reducing oligosaccharides. *o*-Linked glycans also may be released by non-reductive β-elimination. However, one should avoid strong reductive glycan releasing methods using sodium borohydride, as these reduce the C1 aldehyde on the core sugar group and make it unreactive for further derivatization.

Bigge *et al.* (1995) describe a method to label released glycans by reductive amination with the small fluorescent compounds 2-amino benzamide and anthranilic acid (2-aminobenzoic acid). The result is a secondary amine bond with the C1 carbon atom of the reducing end of the glycan (Figure 1.116). The derivatives provide stable fluorescent modifications on glycans, which can be used for detection during electrophoresis, separation, and purification techniques (such as HPLC). The 2-amino benzamide label was found to work best for chromatographic separations, enzymatic modifications, and mass spec analysis, while the anthranilic acid derivative was more suitable for electrophoretic separations, because its negative charge produced sharper bands.

Rothenberg *et al.* (1993) describe the synthesis of a very useful fluorescent glycan labeling agent that contains a biotin group. Diaminopyridine was reacted with sulfo-NHS-biotin to form 2-amino-(6-amidobiotinyl)pyridine (BAP). This reagent contains the fluorescent diaminopyridine group for detection and a biotin handle for purification or immobilization using its strong interaction with streptavidin. BAP can be used to label the reducing ends of glycans and other carbohydrates by reductive amination to form a secondary amine linkage with the aminopyridine group.

The fluorescent 2-aminopyridine group also may be used without a biotin handle for modification of glycans for detection, similar to the use of anthranilic acid and 2-amino benzamide. In this case, the tag just functions as a fluorescent label, which can be used to track glycans during purification or analysis. Modification of glycans with the bifunctional 2,6-diaminopyridine group using reductive amination produces a fluorescently labeled carbohydrate with an available amine for further coupling to surfaces or other biomolecules. Care should be taken, however, when using amine-reactive reagents to modify such amino glycans, because many electrophilic compounds cross-react with hydroxyls on the sugar molecules.

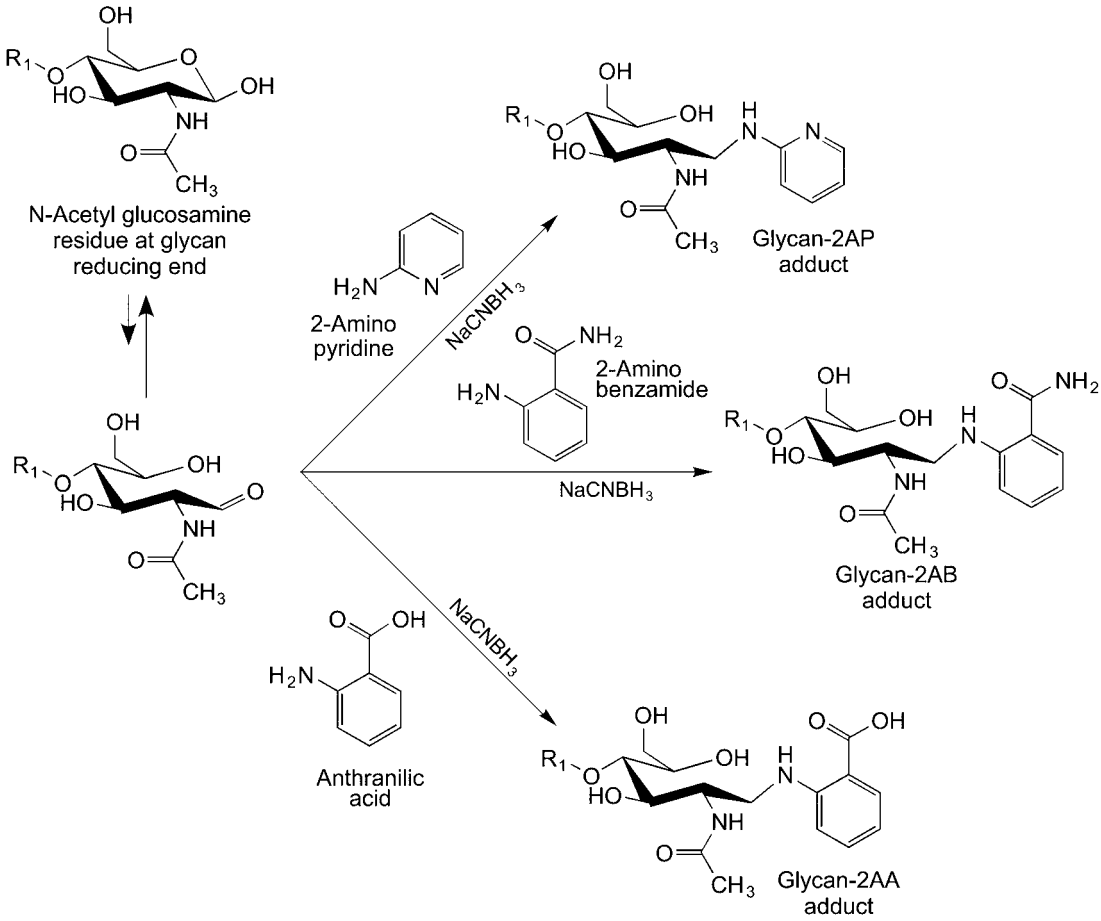


Figure 1.116 Released glycans can be labeled with small fluorescent compounds containing amines for subsequent detection upon chromatographic separation. In the presence of sodium cyanoborohydride these compounds react at the reducing end of glycans to form secondary amine derivatives with characteristic spectral properties.

A similar fluorescent biotin label to the BAP reagent was created by Leteux *et al.* (1998) for labeling glycans without reduction of the reducing end of the monosaccharide. Biotinyl-L-3-(2-naphthyl)-alanine hydrazide (BNAH) contains a strongly UV absorbing naphthalene group, which has fluorescent characteristics, and a hydrazide group for conjugation to the reducing end of glycans. This compound was found to label effectively glycans at the reducing end, while retaining the unreduced nature of the carbohydrate, thus preserving critical protein interactions, which may recognize this region of the sugar.

The reductive amination protocol for coupling these types of small tags to glycans can be found in the previous section.

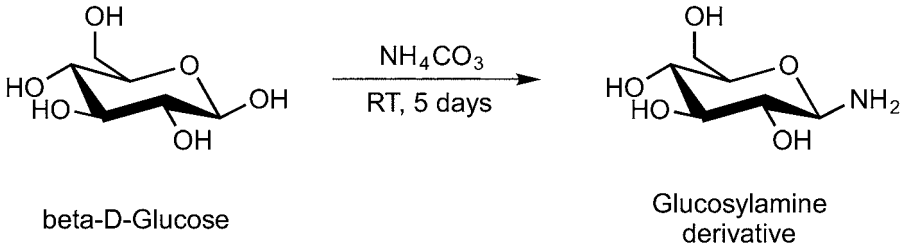


Figure 1.117 Glucosylamine derivatives can be prepared at the reducing end of glycans or other reducing carbohydrates by reaction with ammonium carbonate. The resultant amine derivative can be used to conjugate the carbohydrate with other proteins or molecules without disturbing the cyclic character of the reducing end.

Synthesis of Glycosylamines for Conjugating Glycans

Reducing sugars and carbohydrates or glycans containing an unmodified reducing end may be derivatized in a simple reaction to provide an amine group on C1 for further conjugation. The anomeric hydroxyl group at the reducing end of such sugars can be converted into an amino group by reaction in an aqueous, saturated solution of ammonium carbonate. Kochetkov amination, as it is called (Likhosherstov *et al.*, 1986), can be used to modify a wide variety of reducing carbohydrates and glycans, including neutral and charged monosaccharides, disaccharides, and oligosaccharides (Kallin *et al.*, 1989; Urge *et al.*, 1991, 1992; Manger *et al.*, 1992; Cohen-Anisfeld and Langburg, 1993).

Modification using this protocol yields saccharides that are highly stereochemically pure, with typical reactions giving >95 percent glycosylamines of the β-anomer structure (Figure 1.117). The resulting primary amine group can be further coupled with amine-reactive bioconjugation reagents or used for carbohydrate immobilization onto surfaces or insoluble supports. The major side reaction of amination is the dimeric diglycosylamine derivative, which has two sugar groups attached to a single secondary amine in the middle. This by-product can be present in up to 10 percent of the resulting mass of product formed by the reaction.

The following protocol for synthesis of glycosylamines from reducing sugars is based on the method of Likhosherstov *et al.* (1986).

Protocol

1. Prepare a solution of the saccharide to be aminated at a concentration of up to 1 percent (w/v) in an aqueous solution of saturated ammonium carbonate. Even higher concentrations of saccharides may be used if the carbohydrate being modified is abundant and inexpensive.
2. React with stirring at room temperature for up to 5 days. Over the course of the reaction, add to the solution solid ammonium carbonate at a rate of about 40 mg per mg of saccharide to assure continued saturation.
3. After the reaction, freeze the solution and lyophilize to remove excess ammonium carbonate. Complete removal of volatile salt can be accomplished by re-dissolving the solid in warm methanol. After the completion of CO_2 evolution, dry the saccharide by evaporation under vacuum. Removal of ammonium carbonate is essential, as the ammonium ion will interfere with any subsequent conjugations attempted with the glycosylamine derivative.

5. Blocking Specific Functional Groups

It is often necessary to block specific groups on macromolecules to prevent them from participating in modification or conjugation reactions. In most blocking procedures, a chemical group is covalently coupled to an undesired functional group on the macromolecule to mask or eliminate its reactivity. In this sense, the modification is done with a compound that is relatively inert in whatever application the macromolecule is intended for use. The blocking agent is usually a small organic compound containing a functional group able to couple with the group to be masked. The blocking molecule may contain another functional group of its own, converting the blocked group into a chemical function of another type, but this conversion is all right, providing the newly created function does not interfere in subsequent reactions or applications.

In some cases, a blocking procedure is done to direct a conjugation reaction to discrete sites in a macromolecule. In other instances, blocking a group on one of two macromolecules can prevent self-polymerization and promote the desired intermolecular conjugation. For instance, HRP can be blocked with an amine-specific coupling reagent prior to periodate oxidation to prevent the reactivity of its two amino groups during subsequent conjugation with an antibody molecule (Chapter 20, Section 1.3).

In other uses of blocking reagents, proteins dissociated into subunits by the use of denaturants and disulfide reductants may be prevented from re-association or oxidation of their sulfhydryls by blocking the —SH groups with the appropriate reagent. Alternatively, sulfhydryls may be blocked on a protein prior to activation with a heterobifunctional crosslinking agent that contains both amine-reactive and sulfhydryl-reactive ends. The amine-reactive end will couple to the amines of the protein without reaction of the sulfhydryl-reactive end. This can prevent oligomer formation during the activation process and thus ensure that the sulfhydryl-reactive function is available for conjugation with the desired molecule.

Controlled functional studies of a protein's active center also may be done by blocking specific groups and observing its effect on activity. Often, this blocking procedure is performed through the use of a reversible blocking agent to subsequently regenerate activity; therefore demonstrating the affect was directed at functionalities present in the active site (Perham and Jones, 1967).

Blocking also may be done to quench further modification or conjugation through a targeted functional group. After a conjugation reaction, excess functional groups may be masked from nonspecifically reacting with other molecules. For instance, periodate-oxidized glycoproteins may still contain aldehyde groups after conjugation with another protein by reductive amination. Blocking the aldehydes with a small amine-containing molecule prevents unwanted reactions from occurring when the conjugate is used in an assay or targeting operation. This also is true of excess sulfhydryl groups that may undergo disulfide interchange with other sulfhydryl molecules subsequent to a conjugation reaction. Blocking these groups with the appropriate reagent prevents this type of side reaction from occurring.

Blocking of amine groups on proteins also has been used to create a sensitive reagent for measuring protease activity (Hatakeyama, 1992). With nearly all the primary amines of casein blocked, an amine detection reagent such as TNBS will react only minimally with the protein and form its typical orange derivative. As proteases cleave the protein, however, primary α-amines are created from cleavage of the α-chain peptide bonds, and TNBS then can react with them. The more protease activity present, the more color is formed.

The choice and application of a specific blocking reagent can produce a modified macromolecule with unique and useful properties. Many of the common blocking reagents are discussed

in this section. Beyond the scope of this book, however, is a discussion of the numerous blocking agents used in peptide or nucleic acid synthesis to temporarily block specific reactive groups during growth of the polymer chain.

5.1. Blocking Amine Groups

The amine functionalities most commonly found in macromolecules are primary amines such as those at the N-terminal of polypeptide chains (α-amines) and the side chain ε-amino groups of lysine residues. Several acylation reagents can effectively block these primary amines, some of which are reversible under the right conditions. It should be noted that the cyclic anhydrides mentioned in this section react with amino groups to form amide bonds, opening the anhydride ring and effectively transforming the amine function into a carboxylate. There are additional compounds described in Section 4.2 (this chapter) that also create carboxylates from amines, but in this section the two cyclic anhydrides discussed, maleic anhydride and citraconic anhydride, both are reversible and designed more for temporary masking than permanent blocking. For more stable blocking of amines, the compounds sulfo-NHS acetate and acetic anhydride are the best choices.

Sulfo-NHS Acetate

Sulfo-NHS acetate is the *N*-hydroxysulfosuccinimide ester of acetic acid. The NHS ester end provides high reactivity with the amino groups of proteins at a pH range of 7–9, acylating the amines and forming nonreversible acetamide modifications (Figure 1.118). The sulfonate derivative of the NHS ester provides good water solubility to the reagent. Thus, the compound can be added directly to an aqueous solution of the protein to be blocked, or a stock solution may be prepared and a small aliquot added to the reaction medium. Stock solutions should be dissolved rapidly and used immediately. In aqueous solutions, the main competing reaction is hydrolysis of the active ester to release nonreactive sulfo-NHS and acetic acid. The use of a 10- to 50-fold molar excess of sulfo-NHS acetate over the molar amount of groups to be blocked should provide good yields of acylated amines. Reaction buffers should contain no extraneous amines that could cross-react with the sulfo-NHS acetate. Avoid Tris, glycine, and imidazole containing buffers. Phosphate, borate, or bicarbonate buffers work well at a concentration of 0.05–0.1 M. React for at least 1 hour at room temperature.

It should be noted that complete blocking of all amines on proteins with sulfo-NHS acetate may cause precipitation or loss of native structure and function. The acetate modifications

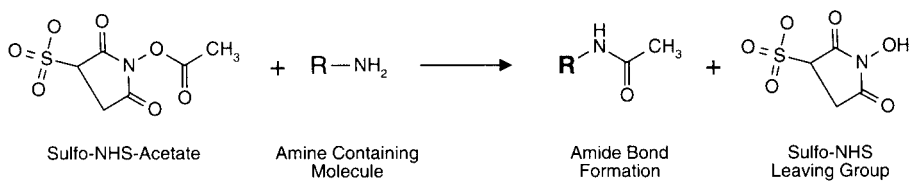
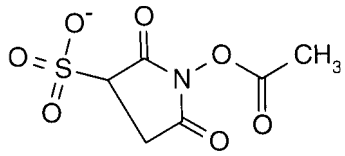


Figure 1.118 Sulfo-NHS acetate may be used to block amine groups, forming permanent amide bond derivatives.

are uncharged and relatively hydrophobic, which may decrease solubility of proteins or other molecules.



Sulfo–NHS-Acetate
MW 259.17

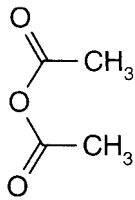
Protocol

1. Dissolve the protein or other amine-containing macromolecule at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5.
2. Add a 25-molar excess of sulfo-NHS acetate over the amount of amines present in the sample. If the precise amount of amines is not known, adding an equal mass of reagent to the mass of protein will provide a large excess of reactivity to completely block all amines.
3. React at room temperature for at least 1 hour.
4. Purify the modified protein by dialysis or gel filtration.

Acetic Anhydride

Acetic anhydride is the only monocarboxylic acid anhydride that is important in modification reactions. The acetylation of the amino groups of proteins can be made relatively specific if the reaction is done in saturated sodium acetate, since the *o*-acetyltyrosine derivative is unstable to an excess of acetate ions (Fraenkel-Conrat, 1959). The tyrosine derivative rapidly hydrolyzes in alkaline reaction conditions, even in the absence of added acetate buffer (Uraki *et al.*, 1957; Smyth, 1967). Treatment with hydroxylamine also cleaves any *o*-acetyltyrosine modifications, forming acetylhydroxamate, which can be followed by its purple complex with Fe^{3+} at 540 nm (Balls and Wood, 1956).

At physiological pH values, acetylation of amine groups proceeds rapidly, requiring less than an hour to go to completion (Figure 1.119).



Acetic Anhydride
MW 102

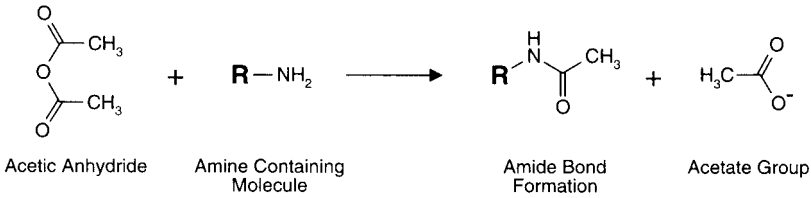


Figure 1.119 Acetic anhydride reacts with amines to form amide bond derivatives.

Protocol

1. Dissolve the macromolecule to be modified at a concentration of 1–10 mg/ml in a buffered solution having a pH between 6.5 and 7.5. Avoid amine-containing buffers such as glycine and Tris. Sodium phosphate buffer at a concentration of 0.1 M works well. The addition of an equal volume of a saturated solution of sodium acetate may be done to prevent tyrosine derivatization.
2. Cool the solution on ice. With stirring, add an amount of acetic anhydride equal to the mass of macromolecule to be modified. Alternatively, add a 10-fold molar excess of acetic anhydride over the amount of amines present. The addition of the anhydride slowly or in several aliquots over the course of 1 hour will assure good yield of acetylation.
3. React with stirring for at least 1 hour while cooling in an ice bath.
4. Purify the acetylated macromolecule by gel filtration or dialysis.

Citraconic Anhydride

Citraconic anhydride (or 2-methylmaleic anhydride) is a derivative of maleic anhydride that is reversible after acylation of amine groups. At alkaline pH values (pH 7–8) the reagent reacts with amines to form amide linkages with an extending terminal carboxylate. However, at acid pH (3–4), these bonds rapidly hydrolyze to release citraconic acid and free the amine (Dixon and Perham, 1968; Habeeb and Atassi, 1970; Klapper and Klotz, 1972; Shetty and Kinsella, 1980). Thus, citraconic anhydride is useful in temporarily blocking amine groups while other parts of a molecule are undergoing derivatization. Once the modification is complete, the amines can be then unblocked to create the original structure. See Section 4.2 (this chapter) for additional information and a protocol for modification of proteins with citraconic anhydride.

Maleic Anhydride

Maleic acid is a linear four carbon molecule with carboxylate groups on both ends and a double bond between the central carbon atoms. The anhydride of maleic acid is a cyclic molecule containing five atoms. Although the reactivity of maleic anhydride is similar to other cyclic anhydrides, the products of maleylation are much more unstable toward hydrolysis, and the site of unsaturation lends itself to additional side reactions. Acylation products of amino groups with maleic anhydride are stable at neutral pH and above, but they readily hydrolyze at acid pH values around pH 3.5 (Butler *et al.*, 1967). Maleylation of sulfhydryls and the phenolate of tyrosine are even more sensitive to hydrolysis. Thus, maleic anhydride is an excellent reversible blocker of amino groups to temporarily mask them from reactivity while another

reaction is being done. For additional information and a protocol for the modification of proteins with this reagent, see Section 4.2 (this chapter).

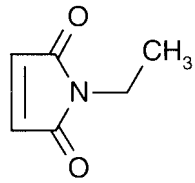
5.2. Blocking Sulfhydryl Groups

The sulfhydryl group is among the most highly reactive of nucleophiles found in biological macromolecules. Cysteine sulfhydryls in proteins undergo covalent reactions rapidly with most of the reactive groups utilized in modification and conjugation reagents. To prevent modification from occurring at these sites, it is often necessary to use a blocking agent that ties up the sulfhydryl and renders it inert toward further reactions.

There are two types of sulfhydryl blocking agents: permanent and reversible. The permanent ones form thioether linkages that don't readily break down. The reversible ones form disulfide bonds that are susceptible to cleavage by the addition of the appropriate reducing agent. Reversible sulfhydryl blockers can be used to temporarily mask an —SH group from modification while a reaction is done at another site. This is especially useful when the sulfhydryl forms a critical part of the active center of a protein. After the final modification is complete, the blocking agent can be removed to regenerate activity.

N-Ethyl Maleimide

N-Ethyl maleimide (NEM) is an alkylating reagent that reacts with sulfhydryls to form stable thioether bonds (Smyth *et al.*, 1960). Maleimide reactions are specific for sulfhydryl groups in the pH range of 6.5–7.5 (Smyth *et al.*, 1964; Gorin *et al.*, 1966; Heitz *et al.*, 1968; Partis *et al.*, 1983) (see Chapter 2, Section 2.2). At higher pH values some cross-reactivity with amino groups takes place (Brewer and Riehm, 1967). One of the carbons adjacent to the double bond undergoes nucleophilic attack by the thiolate anion to generate the addition product (Figure 1.120). When sufficient quantities of —SH groups are being blocked, the reaction may be followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacts and disappears. The result is a stable, inert derivative that terminates in the ethyl group. NEM is useful for permanently blocking sulfhydryl residues in proteins and other macromolecules. It has been used for blocking sulfhydryl-containing reagents that interfere in a glucose oxidase assay system (Haugaard *et al.*, 1981).



N-Ethylmaleimide
MW 125.12

Protocol

1. Dissolve the macromolecule containing sulfhydryl groups to be blocked in a buffer having a pH of 6.5–7.5. Sodium phosphate (0.01–0.1 M) at pH 7.2 works well. Avoid

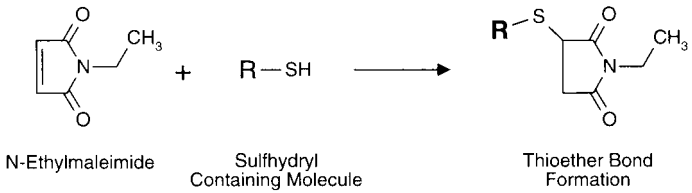


Figure 1.120 The reaction of NEM with sulfhydryl groups yields a thioether derivative, permanently blocking the thiol.

amine-containing buffers, since an excess of amines may cause some reactivity with the maleimide groups. Also, avoid the presence of sulfhydryl containing disulfide reductants such as DTT or 2-mercaptoethanol, which will rapidly react with NEM.

2. Add at least a 10-fold molar excess of NEM over the amount of sulfhydryls present in the reaction. Alternatively, add an equal mass of NEM to the amount of macromolecule present. To facilitate the addition of a small quantity of reagent, a more concentrated stock solution may be prepared in buffer and an aliquot added to the reaction medium. Make the stock solution up fresh, and use it immediately to prevent loss of activity due to maleimide group breakdown.
3. React for 2 hours at room temperature.
4. Purify the modified protein by gel filtration or dialysis.

Iodoacetate Derivatives

Iodoacetate (and bromoacetate) can react with several nucleophilic functional groups within proteins. Their relative reactivity toward protein functionalities is sulfhydryl > imidazolyl > thioether > amine. Among α-haloacetate derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative reactivities, but will obviously not create a charged carboxylate functional group. The acetamide derivatives typically are used only as blocking reagents. The bond formed from the reaction of iodoacetamide and a sulfhydryl group is a stable thioether linkage that is not reversible under normal conditions.

Thus, iodoacetamide has the highest reactivity toward cysteine sulfhydryl residues and may be directed specifically for —SH blocking. If iodoacetamide is present in limiting quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. For additional information on α-haloacetate reactivities and a protocol for blocking, see Section 4.2 (this chapter).

Sodium Tetrathionate

Sodium tetrathionate ($Na_2S_4O_6$) is a redox compound that under the right conditions can facilitate the formation of disulfide bonds from free sulfhydryls. The tetrathionate anion reacts with a sulfhydryl to create a somewhat stable active intermediate, a sulfenylthiosulfate (Figure 1.121). Upon attack of the nucleophilic thiolate anion on this activated species, the thiosulfate ($S_2O_3^-$) leaving group is removed and a disulfide linkage forms (Pihl and Lange, 1962). The

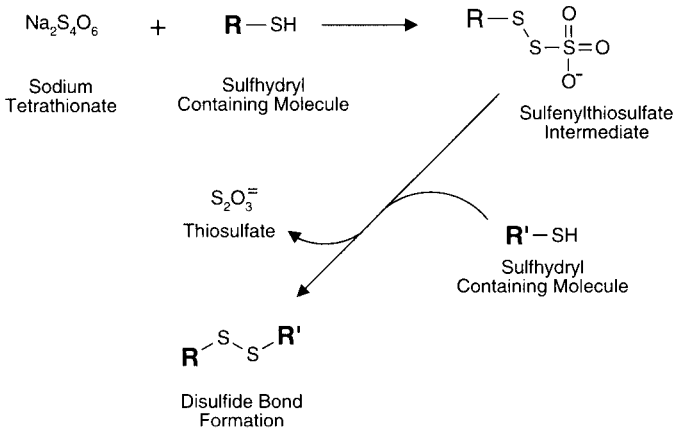


Figure 1.121 Sodium tetrathionate reacts with thiols to form reactive sulfenylthiosulfate intermediates. Another sulfhydryl-containing molecule may couple to this active group to create a disulfide linkage.

reduction of tetrathionate to thiosulfate *in vivo* was a subject of early study (Chen *et al.*, 1934; Theis and Freeland, 1941).

$Na_2S_4O_6$

Sodium Tetrathionate
MW 270.22

Depending on the proximity of cysteine sulfhydryl groups in proteins, intrachain and interchain disulfide formation is possible upon reaction with tetrathionate. When neighboring sulfhydryl groups are not close enough to create disulfide linkages, the sulfenylthiosulfate modification is sufficiently stable to temporarily block exposed —SH groups. For sulfhydryls present in the active centers of enzymes, tetrathionate may lead to reversible inactivation (Parker and Allison, 1969). Thus, the reagent may be used to protect certain sulfhydryl residues during modification reactions performed elsewhere on a protein. Using this approach, the enzyme ficin may be temporarily protected with tetrathionate during modification, conjugation, or immobilization reactions done through its amine groups (Liener and Friedenson, 1970). Subsequent treatment with thiol containing disulfide reducing agents frees the sulfenylthiosulfate and regenerates the sulfhydryl with enzymatic activity. The following protocol is an adaptation of Englund *et al.* (1968), used in the purification of ficin.

Protocol

1. The macromolecule containing sulfhydryl residues to be blocked or protected is dissolved in a buffer suitable for its individual stability requirements. The blocking process may be done on a purified protein or during the early stages of a purification process to protect sulfhydryl active centers from oxidation. PBS buffers containing 1 mM EDTA work well.
2. Add sodium tetrathionate to obtain a final concentration of 10 mM.

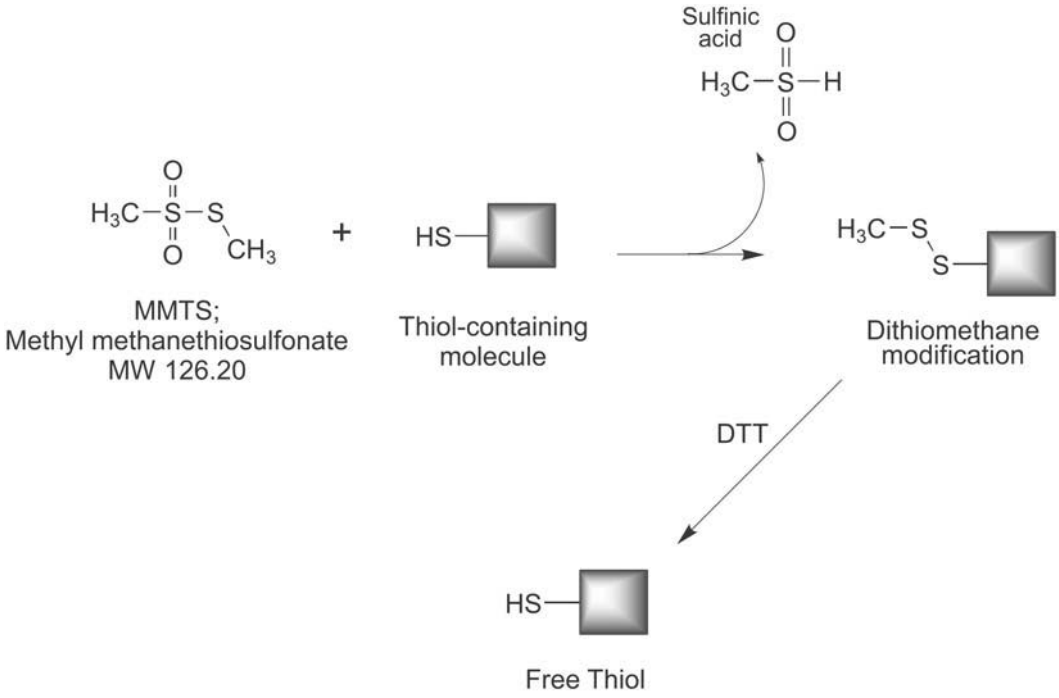


Figure 1.122 MMTS structure and reactions.

3. React for 1 hour at room temperature.
4. Excess tetrathionate may be removed by dialysis or gel filtration.
5. To remove the sulfenylthiosulfate blocking group, add a 300-fold excess of DTT over the amount of blocked sulfhydryls present. Alternatively, add DTT to obtain a 0.01–0.1 M final concentration. Cysteine also may be utilized to regenerate some enzymes to full activity.
6. Incubate for 2 hours at room temperature.
7. For removal of excess DTT, a protein of molecular weight greater than 5,000 may be isolated by gel filtration using a desalting resin. To maintain the stability of the exposed sulfhydryl groups, include 10 mM EDTA in the chromatography buffer. The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm. The protein should elute in the first peak and the DTT reaction products in the second peak.

Methyl Methanethiosulfonate

Methyl methanethiosulfonate (MMTS) is a small reversible blocking agent for sulfhydryl groups (Thermo Fisher, Toronto Research). It reacts with free thiols to form a dithiomethane modification with release of sulfinic acid (Figure 1.122). The sulfinic acid component decomposes into volatile products, which don't affect the disulfide formed from the MMTS reaction Alkylthiosulfonates react rapidly with thiols under mild conditions at physiological pH. The MMTS compound is a liquid at 10.6 M concentration and is conveniently added to a reaction medium by pipette. Complete thiol modifications of available cysteine residues in proteins can

be achieved even using relatively dilute (μM) concentrations of the reagent. Typically, MMTS need only to be added in several- fold molar excess over the quantity of thiols present to results in stoichiometric sulfhydryl group blocking. Reactions can be done in organic solvent, aqueous buffers, or a mixture of organic/aqueous solutions, whatever is suitable for the sulfhydryl compound being modified.

MMTS modifications of thiols are reversiable by use of disulfide reductants. Reducing agents such as DTT, 2-mercaptoethanol, or TCEP will cleave the dithiomethane modification groups to restore the original sulfhydryl. The reagent has been used to identify the cysteine residues important for organic cation transport in oocytes (Sturm *et al*., 2007), to study the peptide loading complex within the MHC class I (Santos *et al*., 2007), for investigations of the Zn^{2+}-dependent redox switch in an intracellular interface channel (Wang *et al*., 2007), and to study how disulfide isomerization functions to switch tissue factor from coagulation to cell signaling (Ahamed *et al*., 2006).

MMTS is a popular thiol blocking agent that functions similar to sodium tetrathionate in forming reversible disulfide derivatives (previous section). This reactive group also has been used as the basis of creating sulfhydryl-reactive crosslinking agents, such as the trifunctional compounds MTS-ATF-Biotin and MTS-ATF-LC-Biotin (Chapter 28, Section 3.2). In addition, it has been used to form thiol modification reagents to study site-directed mutagenesis, including how small modifications might affect protein folding or protein interactions (Toronto Research).

Ellman's Reagent

Ellman's reagent or DTNB, is a compound useful for the quantitative determination of sulfhydryls in solution (Ellman, 1958, 1959). The disulfide of Ellman's reagent readily undergoes disulfide exchange with a free sulfhydryl to form a mixed disulfide and release of one molecule of the chromogenic substance 5-sulfido-2-nitrobenzoate, also called TNB. The intense yellow color produced by the TNB anion can be measured by its absorbance at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$ at pH 8.0). Since each sulfhydryl present generates one molecule of TNB per molecule of Ellman's reagent, direct quantitation is easily done. This reagent has been used to measure the sulfhydryl content in peptides, proteins, and tissue samples (Anderson and Wetlaufer, 1975; Riddles *et al*., 1979). See Section 4.1 in this chapter for the use of Ellman's reagent in the determination of sulfhydryl groups.

The same reaction between Ellman's reagent and the sulfhydryls of macromolecules can be used to temporarily block available —SH groups by the formation of a mixed disulfide bond. Treatment of a sulfhydryl-containing protein with an excess of Ellman's reagent blocks the accessible sulfhydryls with the TNB group, allowing chemistries to be done on other functionalities. Studies have shown that the rate of Ellman's reaction with the sulfhydryl groups in proteins is dependent on their accessibility (Damjanovich and Kleppe, 1966; Colman, 1969). The addition of a disulfide reducing agent then cleaves the TNB group and regenerates the free sulfhydryl. Enzymes containing sulfhydryls in their active sites may be reversibly blocked using this technique to preserve activity after modification or conjugation. Deblocking then restores catalytic activity in most instances.

Protocol

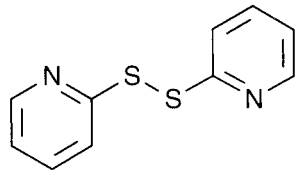
1. Dissolve the protein to be blocked at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, pH 8.0.

2. Dissolve the Ellman's reagent at a concentration of 4 mg/ml in 0.1 M sodium phosphate, pH 8.0.
3. Mix the protein solution with an equal volume of the Ellman's reagent solution and react for 15 minutes at room temperature.
4. Purify the modified protein from excess Ellman's reagent and reaction by-products by dialysis or gel filtration. A measurement of sulfhydryl content may be done by reading the absorbance of the modification reaction at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}cm^{-1}$) versus a series of sulfhydryl standards treated in the same manner (e.g., cysteine).

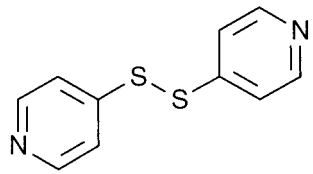
To deblock the TNB-modified sulfhydryl residues, treat the protein with an excess of DTT according to the protocol described in Section 4.1, DTT (this chapter).

Dipyridyl Disulfide Reagents

The similar reagents, 4,4′-dipyridyl disulfide (Grassetti and Murray, 1967) and 2,2′-dipyridyl disulfide (Brocklehurst *et al.*, 1974), react in an analogous manner to Ellman's reagent, both forming pyridyl disulfide bonds with free sulfhydryls and releasing a molecule of either pyridine-4-thione or pyridine-2-thione, respectively (Figure 1.123). Both leaving groups are measurable spectrophotometrically at 324 nm (pyridine-4-thione) or 343 nm (pyridine-2-thione) to quantify the amount of sulfhydryl modification. The reagent 2,2′-dipyridyl disulfide is useful for creating sulfhydryl-reactive crosslinking agents, such as SPDP (Chapter 5, Section 1.1). Both reagents may be used to temporarily block sulfhydryl groups in macromolecules or to activate —SH groups for coupling to another sulfhydryl-containing molecule. The pyridine disulfide-modifying group can react with a sulfhydryl to form a disulfide linkage. The pyridine disulfide also may be cleaved with an excess of disulfide reducing agents, such as DTT, making it a reversible blocking agent.



2,2'-Dipyridyl disulfide



4,4'-Dipyridyl disulfide

Unfortunately, 2,2′-dipyridyl disulfide is relatively insoluble in aqueous buffers. The use of this compound to modify molecules usually involves prior dissolution in an organic solvent

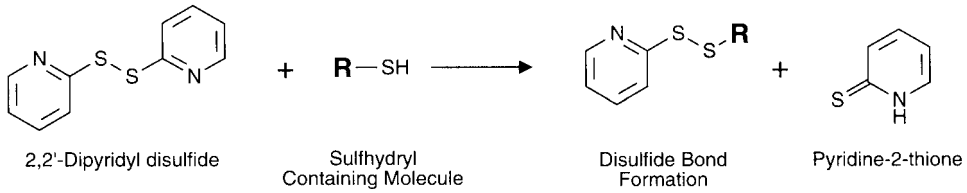


Figure 1.123 2,2′-Dipyridyl disulfide reacts with thiols to form an active pyridyl disulfide intermediate.

such as acetone and then performing the blocking reaction in an aqueous/organic mixture. Many proteins will not tolerate high concentrations of organic solvents without precipitation.

The 4,4′-dipyridyl disulfide can be used in aqueous solutions, but it has been found that modification of proteins with this reagent yields rapid disulfide bond formation. Only when 2-iminothiolane is used in tandem with 4,4′-dipyridyl disulfide can 4-dithiopyridyl groups be introduced into proteins (King *et al.*, 1978) (Section 4.1, this chapter). This is due to disulfide interchange reactions predominating without the addition of 2-iminothiolane.

For one-step methods, the use of Ellman's reagent (previous section) to yield a similar reversible sulfhydryl blocking group is probably a better choice with protein molecules.

5.3. Blocking Aldehyde Groups

Aldehyde groups are useful in facilitating modification or conjugation reactions, easily forming secondary amine linkages with amine-containing molecules in reductive amination procedures or hydrazone linkages with hydrazide-containing molecules. Macromolecules modified to contain aldehyde groups for use in these reactions (see Section 4.4, this chapter) should be treated after conjugation to remove any excess formyl functionalities. The blocking step prevents subsequent nonspecific interactions when a conjugate is used in assay or targeting applications.

Reductive Amination with Tris or Ethanolamine

The simplest method for blocking aldehyde functionalities involves reductive amination with a small amine-containing molecule. The best such blockers do not have extra functionalities that may create additional sites of reactivity after blocking. Tris and ethanolamine are ideal in this regard. They both contain primary amines that readily react with aldehydes in the presence of a reductant, and they both possess relatively inert hydroxyl groups that maintain hydrophilicity after coupling. Reductive amination (Chapter 1, Section 5.3 and Chapter 3, Section 4) facilitated by the use of sodium cyanoborohydride can quickly block residual aldehyde groups and transform them into unreactive hydroxyls of low nonspecific binding potential (Figure 1.124).

Protocol

1. Dissolve the macromolecule-containing aldehydes to be blocked (i.e., a glycoprotein that has been oxidized with sodium periodate to create formyl groups) at a concentration

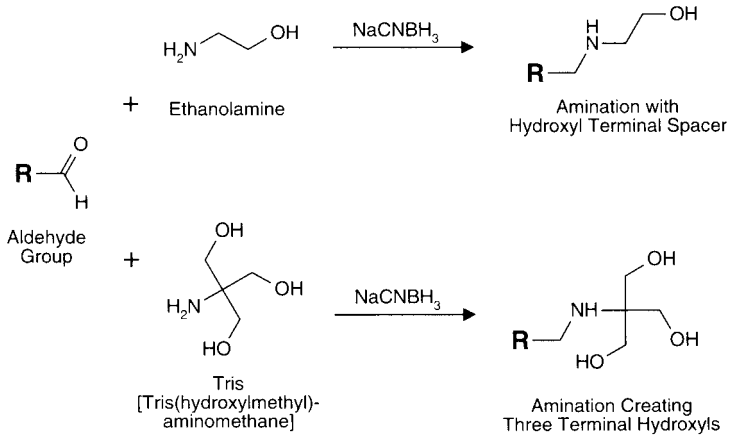


Figure 1.124 Aldehyde groups may be blocked with Tris or ethanolamine using a reductive amination process.

of 1–10 mg/ml in 0.1 M Tris buffer, pH 8.0. Alternatively, dissolve the macromolecule in 0.1 M sodium phosphate containing 0.1 M ethanolamine, pH 8.0. The use of other buffers having a pH between 7 and 10 will work as well, but the Tris or ethanolamine concentrations should be maintained in high excess to efficiently block all the aldehyde residues.

2. Add 10 µl of 5 M sodium cyanoborohydride in 1 N NaOH (Aldrich) per ml of the macromolecule solution volume prepared in (1). *Caution*: Highly toxic compound. Use a fume hood and be careful to avoid skin contact with this reagent.
3. React for 15 minutes at room temperature.
4. Purify the derivatized macromolecule by dialysis or gel filtration using a buffer suitable for the nature of the substance being modified.

5.4. Blocking Carboxylate Groups

The presence of unwanted carboxylate groups in macromolecules may be easily blocked by the use of a small amine-containing molecule coupled via the carbodiimide procedure (Chapter 3, Section 1).

Tris or Ethanolamine Plus EDC

Tris or ethanolamine are excellent choices for blocking procedures involving carboxylic acid groups, since they contain hydrophilic hydroxyls that mask the carboxylate and create an inert modification with low nonspecific binding potential. Using the water-soluble carbodiimide EDC to facilitate this reaction, the carboxylate is activated by forming an intermediate *o*-acylisourea. The amine-containing compound then reacts with this active species to create a stable amide linkage (Figure 1.125).

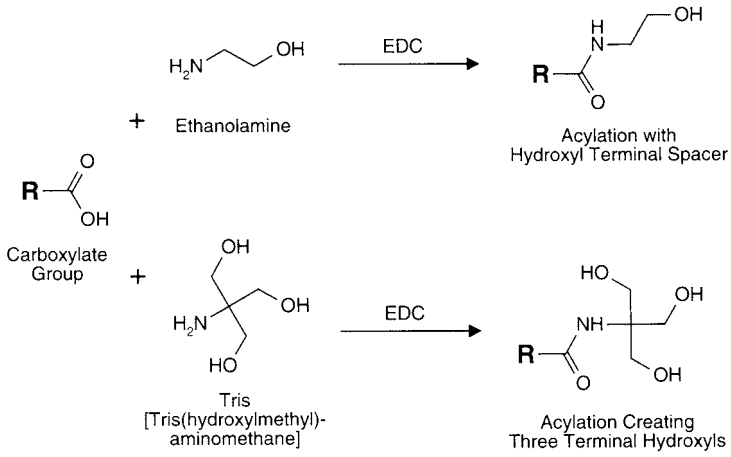


Figure 1.125 Carboxylate groups may be blocked with Tris or ethanolamine using a carbodiimide-mediated process.

Protocol

1. Dissolve the macromolecule containing carboxylate groups to be blocked at a concentration of 1–10 mg/ml in 0.1 M MES, pH 4.7, containing 0.1 M Tris or ethanolamine. Other conditions may be used to perform this reaction. See Chapter 3, Section 1 for further details.
2. Add 10 mg of EDC per ml of the solution prepared in (1).
3. React for 2–4 hours at room temperature.
4. Purify by gel filtration or dialysis.

2

The Chemistry of Reactive Groups

Every chemical modification or conjugation process involves the reaction of one functional group with another, resulting in the formation of a covalent bond. The creation of bioconjugate reagents with spontaneously reactive or selectively reactive functional groups forms the basis for simple and reproducible crosslinking or tagging of target molecules. Of the hundreds of reagent systems described in the literature or offered commercially, most utilize common organic chemical principles that can be reduced down to a couple dozen or so primary reactions. An understanding of these basic reactions can provide insight into the properties and use of bioconjugate reagents even before they are applied to problems in the laboratory.

This section is designed to provide a general overview of activation and coupling chemistry. Some of the reagents discussed in this chapter are not themselves crosslinking or modification compounds, but may be used to form active intermediates with another functional group. These active intermediates subsequently can be coupled to a second molecule that possesses the correct chemical constituents, which allows bond formation to occur.

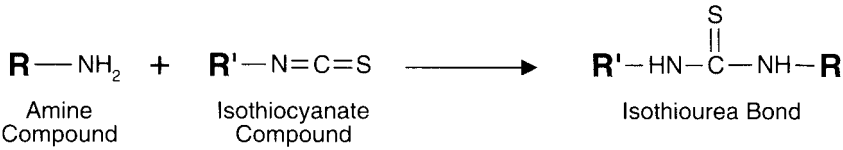
Ultimately, this section is meant to function as a ready-reference database for learning or review of bioconjugate chemistry. In this regard, a reaction can be quickly found, a short discussion of its properties and use read, and a visual representation of the chemistry of bond formation illustrated. What this section is not meant to be is an exhaustive discussion on the theory or mechanism behind each reaction, nor a review of every application in which each chemical reaction has been used. For particular applications where the chemistries are employed, cross-references are given to other sections in this book or to outside literature sources.

1. Amine Reactions

Reactive groups able to couple with amine-containing molecules are by far the most common functional groups present on crosslinking or modification reagents. An amine-coupling process can be used to conjugate with nearly all protein or peptide molecules as well as a host of other macromolecules. The primary coupling reactions for modification of amines proceed by one of two routes: acylation or alkylation (Chapter 1, Section 1.1). Most of these reactions are rapid and occur in high yield to give stable amide or secondary amine bonds.

1.1. Isothiocyanates

Isothiocyanates can be formed by the reaction of an aromatic amine with thiophosgene (Rifai and Wong, 1986). The group reacts with nucleophiles such as amines, sulfhydryls, and the phenolate ion of tyrosine side chains (Podhradsky *et al.*, 1979). The only stable product of these reactions, however, is with primary amine groups. Therefore, isothiocyanate compounds are almost entirely selective for modifying ε-amino groups in lysine side chains and N-terminal α-amines in proteins or primary amines in other molecules (Jobbagy and Kiraly, 1966). The reaction involves attack of the nucleophile on the central, electrophilic carbon of the isothiocyanate group (Reaction 1). The resulting electron shift and proton loss creates a thiourea linkage between the isothiocyanate-containing compound and the amine with no leaving group involved.

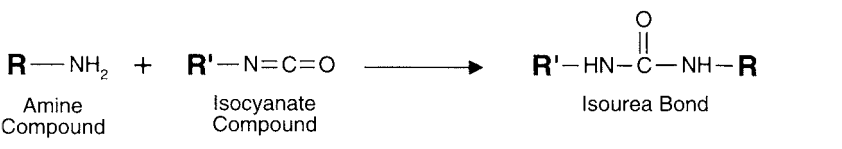


(Reaction 1)

Isothiocyanate compounds react best at alkaline pH values where the target amine groups are mainly unprotonated. Many reactions are done in 0.1 M sodium carbonate buffer at pH 9.0. Reaction times vary from 4 to 24 hours at 4°C. Rana and Meares (1990) found that by reacting isothiocyanate-containing chelates at pH 7 they could selectively modify a monoclonal antibody only at its N-terminal α-amines while leaving lysine amines unmodified. This is an excellent method for selectively modifying only a single site on a protein or peptide molecule. Since the isothiocyanate group is relatively unstable in aqueous conditions, reagents containing this function should be stored desiccated at refrigerator or freezer temperatures.

1.2. Isocyanates

Isocyanates are similar to the isothiocyanates discussed above, except an oxygen atom replaces the sulfur. An isocyanate can be formed from the reaction of an aromatic amine with phosgene (Rifai and Wong, 1986). The group also can be created from acyl azides by treatment at 80°C in the presence of an alcohol (Section 4.7, this chapter). Under these conditions, the acyl azide rearranges to form an isocyanate. Isocyanates can react with amine-containing molecules to form stable isourea linkages (Reaction 2). The reactivity of isocyanates is greater than that of isothiocyanates, but for the same reason their stability can be a problem. Many commercial suppliers of bioconjugate reagents have found isocyanate compounds too unstable to offer them for sale, since moisture rapidly decomposes them, releasing CO_2 and leaving an aromatic amine.

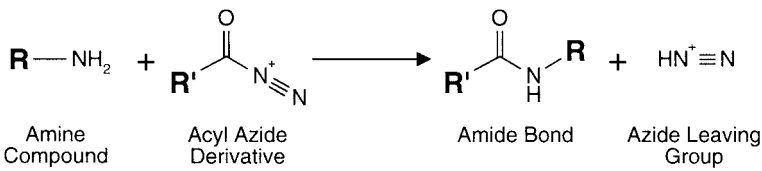


(Reaction 2)

Isocyanate-containing reagents also can be used to crosslink or label hydroxyl-containing molecules. Recently, a heterobifunctional compound containing a isocyanate group on one end and a maleimide group on the other end was reported (Annunziato *et al.*, 1993). PMPI, or *p*-maleimidophenyl isocyanate, can be used to conjugate hydroxyl-containing compounds such as polysaccharides with sulfhydryl-containing molecules (available from Thermo Fisher).

1.3. Acyl Azides

Acyl azides are activated carboxylate groups that can react with primary amines to form amide bonds. The azide function is a good leaving group similar to the *N*-hydroxysuccinimide group of NHS ester compounds. An acyl azide can be formed by treatment of a hydrazide with sodium nitrite at 0°C (Lowe and Dean, 1974). A coupling reaction with an amine group occurs by attack of the nucleophile at the electron-deficient carbonyl group (Reaction 3). Optimum conditions for the reaction are a pH range of 8.5–10 in buffers which contain no competing amines or other nucleophiles.



(Reaction 3)

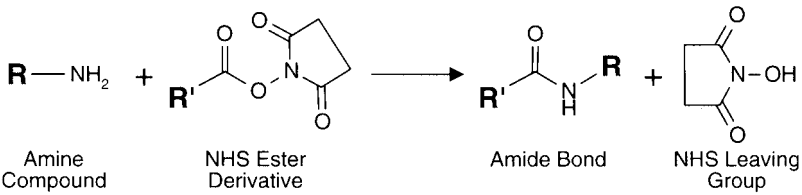
The major competing reaction in acyl azide coupling is hydrolysis. The higher the pH, the faster the reactivity, both with regard to amine conjugation and hydrolysis. Crosslinkers or modification reagents containing this compound must be kept dry to preserve activity. Reactions are complete in 2–4 hours at room temperature.

1.4. NHS Esters

An *N*-hydroxysuccinimide (NHS) ester is perhaps the most common activation chemistry for creating reactive acylating agents. NHS esters were first introduced as reactive ends of homobifunctional crosslinkers (Bragg and Hou, 1975; Lomant and Fairbanks, 1976). Today, the great majority of amine-reactive crosslinking or modification reagents commercially available utilize NHS esters. An NHS ester may be formed by the reaction of a carboxylate with NHS in the presence of a carbodiimide. To prepare stable NHS ester derivatives, the activation reaction must be done in non-aqueous conditions using water-insoluble carbodiimides or condensing agents, such as DCC (Chapter 3, Section 1.4).

NHS or sulfo-NHS ester-containing reagents react with nucleophiles with release of the NHS or sulfo-NHS leaving group to form an acylated product (Reaction 4). The reaction of such esters with a sulfhydryl or hydroxyl group does not yield stable conjugates, forming thioesters or ester linkages, respectively. Both of these bonds potentially can hydrolyze in aqueous environments or exchange with neighboring amines to form amide bonds. Histidine

side-chain nitrogens of the imidazolyl ring also may be acylated with an NHS ester reagent, but they hydrolyze very rapidly in aqueous environments (Cuatrecasas and Parikh, 1972). Thus, the presence of imidazole in reaction buffers only serves to increase the hydrolysis rate of the active ester. Reaction with primary and secondary amines, however, creates stable amide and imide linkages, respectively, that don't readily break down. Thus, in protein molecules, NHS ester crosslinking reagents couple principally with the α-amines at the N-terminals and the ε-amines of lysine side chains.



(Reaction 4)

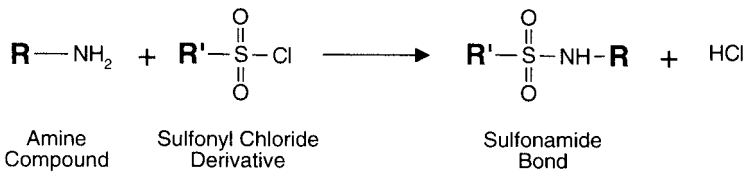
NHS esters also may be formed *in situ* to react immediately with target molecules in aqueous reaction media. Using the water-soluble carbodiimide EDC (Chapter 3, Section 1.1) a carboxylate-containing molecule can be transformed into an active ester by reaction in the presence of NHS or sulfo-NHS (*N*-hydroxysulfosuccinimide) (Chapter 3, Section 1.2). Sulfo-NHS esters are hydrophilic active groups that couple rapidly with amines on target molecules with the same specificity and reactivity as NHS esters (Staros, 1982). Unlike NHS esters that are relatively water insoluble and must be first dissolved in organic solvent before being added to aqueous solutions, sulfo-NHS esters are relatively water soluble, longer-lived, and hydrolyze more slowly in water. In the presence of amine nucleophiles that can attack at the electron-deficient carbonyl of the active ester, the sulfo-NHS group rapidly leaves, creating a stable amide linkage with the amine compound. Sulfhydryl and hydroxyl groups also will react with such active esters, but the products of such reactions, thioesters and esters, are unstable in aqueous environments or in the presence of amine nucleophiles.

NHS esters have a half-life on the order of hours under physiological pH conditions. However, hydrolysis and amine reactivity both increase with increasing pH. At 0°C at pH 7.0, the half-life is typically 4–5 hours (Lomant and Fairbanks, 1976). At pH 8.0 at 25°C it falls to 1 hour (Staros, 1988), and at pH 8.6 and 4°C the half-life is only 10 minutes (Cuatrecasas and Parikh, 1972). The rate of hydrolysis may be monitored by measuring the increase in absorptivity at 260 nm as the NHS leaving group is cleaved. The molar extinction coefficient of the NHS group in solution is $8.2 \times 10^3/M^{-1}cm^{-1}$ in Tris buffer at pH 9.0 (Carlsson *et al.*, 1978), but somewhat decreases to $7.5 \times 10^3/M^{-1}/cm^{-1}$ in potassium phosphate buffer at pH 6.5 (Partis *et al.*, 1983). Unfortunately, the relatively low sensitivity of this absorptivity measurement does not allow for determining the rate of reaction in an actual crosslinking procedure.

To maximize the modification of amines and minimize the effects of hydrolysis, maintain a high concentration of protein or other target molecule in the reaction medium. By adjusting the molar ratio of crosslinker to target molecule(s), the level of modification and conjugation may be controlled to create an optimal product. Water-insoluble crosslinkers containing NHS esters may be reacted in organic solvents, eliminating the hydrolysis problem, provided the target molecule is soluble and stable in such environments. For non-aqueous reactions, an organic base (proton acceptor) typically is added, such as triethylamine or 4-(dimethylamine)pyridine (DMAP).

1.5. Sulfonyl Chlorides

Sulfonyl chlorides are reactive sulfonic acid derivatives similar in properties and reactivity to acid chlorides of carboxylates. The sulfonic acid group, however, is a highly hindered molecule, containing a tetrahedral configuration of substituents. The attack of a nucleophile on a sulfonyl chloride involves temporary formation of a pentavalent intermediate which is highly crowded and unstable. Unlike the capability of using other condensing agents such as carbodiimides when preparing amide linkages between carboxylate groups and amines, sulfonic acids are too hindered to allow such bulky active intermediates to be formed. Thus, the main activation chemistry employed with sulfonates is to create the sulfonyl chloride derivative. Reaction of a sulfonyl chloride compound with a primary amine-containing molecule proceeds with loss of the chlorine atom and formation of a sulfonamide linkage (Reaction 5).



(Reaction 5)

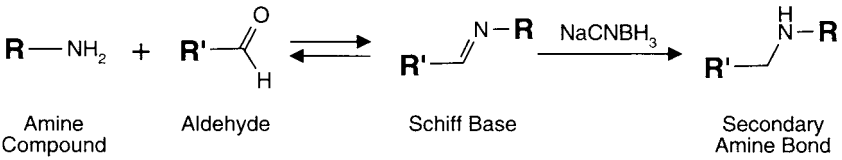
Sulfonic acids are frequent constituents of fluorescent probes (Chapter 9, Section 1). The sulfonyl chloride derivative allows simple conjugation of these molecules with proteins and other amine-containing compounds. The derivative is prepared by reaction of the sulfonate with thionyl chloride or phosphorus pentachloride in non-aqueous conditions. The reaction of a sulfonyl chloride with an amine proceeds under alkaline pH conditions (typically done at pH 9–10). It may also be done in organic solvent for the modification of water-insoluble compounds. Hydrolysis is the major competing reaction in aqueous environments, although the overall rate of sulfonyl chloride reactivity and hydrolysis is less than that of the corresponding acid chlorides of carboxylates. However, sulfonyl chloride-containing reagents should be stored under nitrogen or in a desiccator to prevent breakdown by moisture.

1.6. Aldehydes and Glyoxals

Carbonyl groups such as aldehydes, ketones, and glyoxals can react with amines to form Schiff base intermediates which are in equilibrium with their free forms. The interaction is pH dependent, being more efficient at low pH and especially efficient at high pH conditions. Certain compounds, particularly some reducing sugars, may undergo an Amadori rearrangement after Schiff base formation to a stable ketoamine structure (Chapter 1, Section 2.1). This occurs *in vivo* as glucose modifies amine-containing components in the blood to form glycated derivatives. Such modification is thought to be related to aging and is a signal for protein and cellular regeneration.

The rather labile Schiff base interaction can be chemically stabilized by reduction. The addition of sodium borohydride or sodium cyanoborohydride to a reaction medium containing an aldehyde compound and an amine-containing molecule will result in reduction of the Schiff

base intermediate and covalent bond formation, creating a secondary amine linkage between the two molecules (Reaction 6).

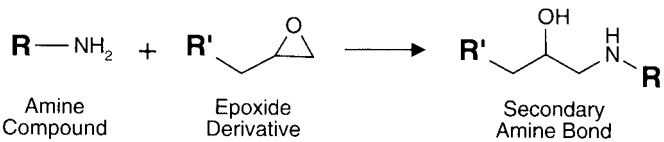


(Reaction 6)

Although both borohydride and cyanoborohydride have been used for reductive amination purposes, borohydride will reduce the reactive aldehyde groups to hydroxyls at the same time it converts any Schiff bases present to secondary amines. Cyanoborohydride, by contrast, is a milder reducing agent. It has been shown to be at least 5 times milder than borohydride in reductive amination processes with antibodies (Peng *et al.*, 1987). While cyanoborohydride does not reduce aldehydes, it is very effective at Schiff base reduction. Thus, higher yields of conjugate formation can be realized using cyanoborohydride instead of borohydride. Other reducing agents also have been explored for reductive amination processes, including various amine boranes and ascorbic acid (Cabacungan *et al.*, 1982; Hornsey *et al.*, 1986). See Chapter 3, Section 4 for a protocol for reductive amination coupling.

1.7. Epoxides and Oxiranes

An epoxide or oxirane group will react with nucleophiles in a ring-opening process. The reaction can take place with primary amines, sulfhydryls, or hydroxyl groups to create secondary amine, thioether, or ether bonds, respectively. During the coupling process, ring opening forms a β-hydroxy group on the epoxy compound (Reaction 7). The reaction of the epoxide functionalities with hydroxyls requires high pH conditions, usually in the range of pH 11–12. Amine nucleophiles react at more moderate alkaline pH values, typically needing buffer environments of at least pH 9.0. Sulfhydryl groups are the most highly reactive nucleophiles with epoxides, requiring a buffered system closer to the physiological pH range of 7.5–8.5 for efficient coupling.

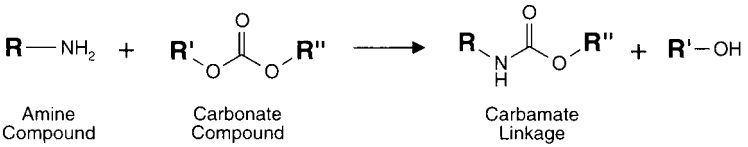


(Reaction 7)

The principal side reaction to epoxide coupling is hydrolysis. Particularly at acid pH values, the epoxide ring can hydrolyze to form adjacent hydroxyls. This diol can be oxidized with periodate to create a terminal aldehyde residue with loss of one molecule of formaldehyde (Chapter 1, Section 4.4). The aldehyde then can be used in reductive amination reactions. The reaction of an epoxide group with an ammonium ion generates a terminal primary amine group that also can be used for further derivatization.

1.8. Carbonates

Carbonates are diester derivatives of carbonic acid formed from its condensation with hydroxyl compounds. These groups may be created from the reaction of a bifunctional carbonic acid compound like phosgene or carbonyldiimidazole (CDI) (Chapter 3, Section 3) with two alcohols. Carbonates can rapidly react with nucleophiles to form carbamate linkages, which are extremely stable bonds (Reaction 8). A commonly used bifunctional carbonate compound, disuccinimidyl carbonate, can be used to activate hydroxyl-containing molecules to form amine-reactive succinimidyl carbonate intermediates (Section 4.3, this chapter). This carbonate activation procedure can be used with great success in coupling polyethylene glycol (PEG) to proteins and other amine-containing molecules (Chapter 25, Section 1.2).



(Reaction 8)

Nucleophiles, such as the primary amino groups of proteins, can react with the succinimidyl carbonate functional groups to give stable carbamate (aliphatic urethane) bonds. The linkage is identical to that obtained through CDI activation of hydroxyl groups with subsequent coupling of amines (Chapter 3, Section 3 and Chapter 25, Section 1.4). However, the reactivity of the succinimidyl carbonate is much greater than that of the imidazole carbamate formed as the active species in CDI activation. A succinimidyl carbonate group may hydrolyze in aqueous solution to release NHS and CO_2, essentially regenerating the underivatized hydroxyl. Carbonates formed from esterification of two alcohol groups similarly hydrolyze to release CO_2 plus the original hydroxyl compounds.

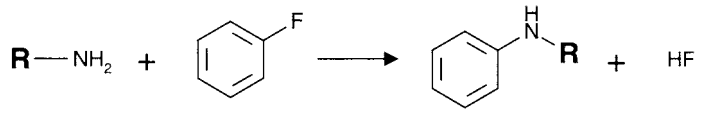
The coupling reaction of a carbonate functional group with an amine is best done in slightly alkaline pH (7–9) and in the absence of any competing amine or sulfhydryl components.

1.9. Arylating Agents

Aryl halide compounds such as fluorobenzene derivatives can be used to form covalent bonds with amine-containing molecules like proteins. The reactivity of aryl halides, however, is not totally specific for amines. Other nucleophiles such as thiol, imidazolyl, and phenolate groups of amino acid side chains also can react (Zahn and Meinhoffer, 1958). Conjugates formed with sulfhydryl groups are reversible by cleaving with an excess of thiol (Shaltiel, 1967).

Fluorobenzene-type compounds have been used as functional groups in homobifunctional crosslinking agents (Chapter 4, Section 4). Their reaction with amines involves nucleophilic displacement of the fluorine atom with the amine derivative, creating a substituted aryl amine bond (Reaction 9). Detection reagents incorporating reactive aryl chemistry include 2,4-dinitrofluorobenzene and trinitrobenzenesulfonate (Eisen *et al.*, 1953). These compounds form

colored complexes with target amine groups. The relative rate of reactivity for aryl compounds is: F > Cl ~ Br > Sulfonate.

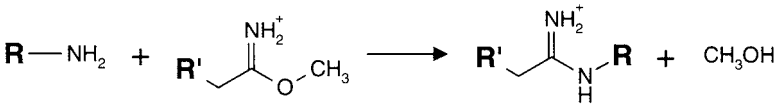


Amine Compound | Fluorobenzene Derivative | Arylamine Bond

(Reaction 9)

1.10. Imidoesters

The Imidoester (or imidate) functional group is one of the most specific acylating agents available for modifying primary amines. Unlike most other coupling chemistries, imidoesters possess minimal cross-reactivity toward other nucleophilic groups in proteins. The α-amines and ε-amines of proteins may be targeted and crosslinked by reacting with homobifunctional imidoesters at a pH of 7–10 (optimal pH 8–9). The product of this reaction, an imidoamide (or amidine) (Reaction 10), is protonated and thus carries a positive charge at physiological pH (Kiehm and Ji, 1977; Liu *et al.*, 1977; Ji, 1979; Wilbur, 1992).



Amine Compound | Imidoester Compound | Amidine Linkage

(Reaction 10)

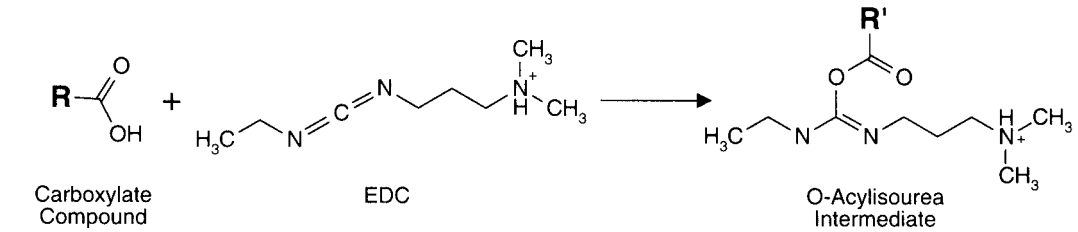
The amidine bond formed is quite stable at acid pH; however, it is susceptible to hydrolysis and cleavage at high pH. A typical reaction condition for using imidate crosslinkers is a buffer system consisting of 0.2 M triethanolamine in 0.1 M sodium borate, pH 8.2. After conjugating two proteins with a bifunctional imidoester crosslinker, excess imidoester functional groups may be blocked with ethanolamine.

1.11. Carbodiimides

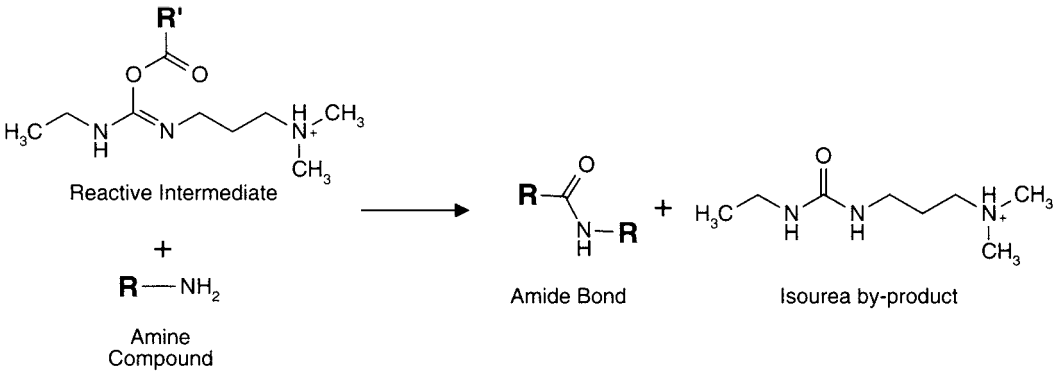
Carbodiimides are zero-length crosslinking agents used to mediate the formation of an amide or phosphoramidate linkage between a carboxylate group and an amine or a phosphate and an amine, respectively (Hoare and Koshland, 1966; Chu *et al.*, 1986; Ghosh *et al.*, 1990). They are called zero-length reagents because in forming these bonds no additional chemical structure is introduced between the conjugating molecules.

N-substituted carbodiimides can react with carboxylic acids to form highly reactive, *o*-acylisourea derivatives that are extremely short-lived (Reaction 11). This active species then can react with a nucleophile such as a primary amine to form an amide bond (Reaction 12)

(Williams and Ibrahim, 1981). Other nucleophiles also are reactive. Sulfhydryl groups may attack the active species and form thioester linkages, although these are not as stable as the bond formed with an amine.



(Reaction 11)



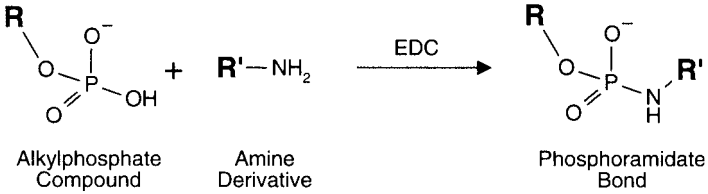
(Reaction 12)

Hydrazide-containing compounds also can be coupled to carboxylate groups using a carbodiimide-mediated reaction. Using bifunctional hydrazide reagents, carboxylates can be modified to possess terminal hydrazide groups able to conjugate with other carbonyl compounds (Chapter 4, Section 8).

In addition, oxygen atoms may act as the attacking nucleophile, such as those in water molecules. In aqueous solution, hydrolysis by water is the major competing reaction, both inactivating EDC itself and cleaving off the activated ester intermediate, forming an isourea, and regenerating the carboxylate group (Gilles *et al.*, 1990).

Nakajima and Ikada (1995) investigated the reactions of EDC amide bond formation in aqueous solution using a hydrogel of poly(acrylic acid) to contribute the carboxylate groups and ethylenediamine or benzylamine as the amine functional groups. Their results indicate that carboxylate activation occurs most effectively at pH 3.5–4.5, while amide bond formation occurs with highest yield at pH 4–6. However, the maximal rate of hydrolysis of EDC occurs at acidic pH values with increasing stability of the carbodiimide in solution at or above pH 6.5. When working with proteins and peptides, experience indicates that EDC-mediated amide bond formation effectively occurs between pH 4.5 and 7.5. Buffer systems using MES or phosphate may be used to stabilize the pH during the course of the reaction. For additional information on specific carbodiimides used in bioconjugate chemistry, see Chapter 3, Section 1.

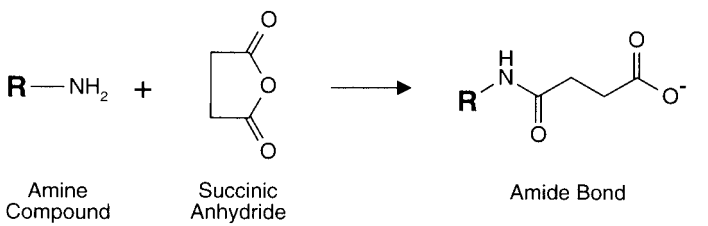
Molecules containing phosphate groups, such as the 5′ phosphate of oligonucleotides, also may be conjugated to amine-containing molecules by using a carbodiimide-mediated reaction (Chapter 27, Section 2.1). The carbodiimide activates the phosphate to an intermediate phosphate ester similar to its reaction with carboxylates (Chapter 3, Section 1). In the presence of an amine, the ester reacts to form a stable phosphoramidate bond (Reaction 13).



(Reaction 13)

1.12. Anhydrides

Acid anhydrides, as their name implies, are formed from the dehydration reaction of two carboxylic acid groups. Anhydrides are highly reactive toward nucleophiles and are able to acylate a number of the important functional groups of proteins and other macromolecules. Upon nucleophilic attack, the anhydride yields one carboxylic acid for every acylated product. If the anhydride was formed from monocarboxylic acids, such as acetic anhydride, then the acylation occurs with release of one carboxylate group. However, for dicarboxylic acid anhydrides, such as succinic anhydride, upon reaction with a nucleophile the ring structure of the anhydride opens, forming the acylated product modified to contain a newly formed carboxylate group (Reaction 14). Thus, anhydride reagents may be used to both block functional groups and to convert an existing functional group into a carboxylic acid.



(Reaction 14)

Protein functional groups able to react with anhydrides include the α-amines at the N-terminals, the ε-amine of lysine side chains, cysteine sulfhydryl groups, the phenolate ion of tyrosine residues, and the imidazolyl ring of histidines. However, acylation of cysteine, tyrosine, and histidine side chains forms unstable complexes that are easily reversible to regenerate the original group. Only amine functional groups of proteins are stable to acylation with anhydride reagents, forming amide bonds (Fraenkel-Conrat, 1959; Smyth, 1967).

Another potential site of reactivity for anhydrides in protein molecules is modification of any attached carbohydrate chains. In addition to amino group modification in the polypeptide chain, glycoproteins may be modified at their polysaccharide hydroxyl groups to form esterified

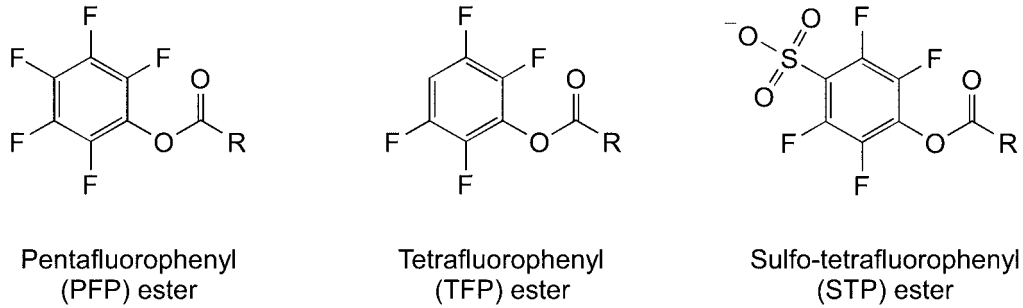


Figure 2.1 Three types of fluorophenyl esters have been used for coupling to amine-containing molecules. The PFP and TFP esters are relatively hydrophobic and typically have better stability toward hydrolysis in aqueous solution than NHS esters. The STP ester is water-soluble due to the negatively charged sulfonate group, and it provides better solubility to associated crosslinkers or bioconjugation reagents similar to that of a sulfo-NHS ester group.

derivatives. Esterification of carbohydrates by acetic anhydride, especially cellulose, is a major industrial application for this compound. In aqueous solutions, however, esterification will be a minor product, since the oxygen of water is about as strong a nucleophile as the hydroxyls of sugar residues.

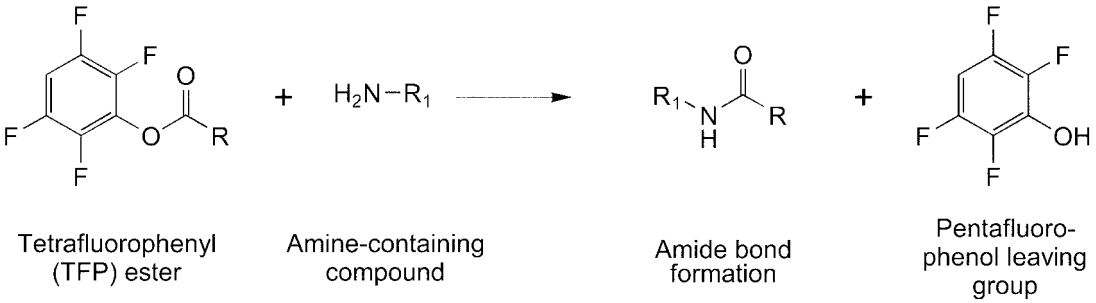
The major side reaction to the desired acylation product is hydrolysis of the anhydride. In aqueous solutions, anhydrides may breakdown by the addition of one molecule of water to yield two unreactive carboxylate groups. The presence of an excess of the anhydride in the reaction medium usually is used to minimize the effects of competing hydrolysis.

Since both hydrolysis and acylation result in the release of carboxylic acid functionalities, the medium becomes acidic during the course of the reaction. This requires either the presence of a strongly buffered environment to maintain the pH or periodic monitoring and adjustment of the pH with base as the reaction progresses.

1.13. Fluorophenyl Esters

Another type of carboxylic acid derivative that reacts with amines consists of the ester of a fluorophenol compound, which creates a group capable of forming amide bonds with proteins and other molecules. Several types of fluorophenyl esters have been used as reactive groups: a pentafluorophenyl (PFP) ester, a tetrafluorophenyl (TFP) ester, and a sulfo-tetrafluorophenyl (STP) ester. All of these derivatives have similar reactivity with amines, but the uncharged ones are hydrophobic and the sulfonated one is negatively charged in aqueous solution, thus providing water solubility to active ester compounds (Gee *et al.*, 1999) (Figure 2.1).

Fluorophenyl esters react with amine-containing molecules at slightly alkaline pH values to give the same amide bond linkages as NHS esters (Reaction 15). However, in most cases, the fluorophenyl ester compound will display better stability toward hydrolysis in aqueous solution. It has been reported that a TFP ester has over twice the half-life in basic pH buffers (pH ~8) than a corresponding NHS ester on the same compound (Molecular Probes).

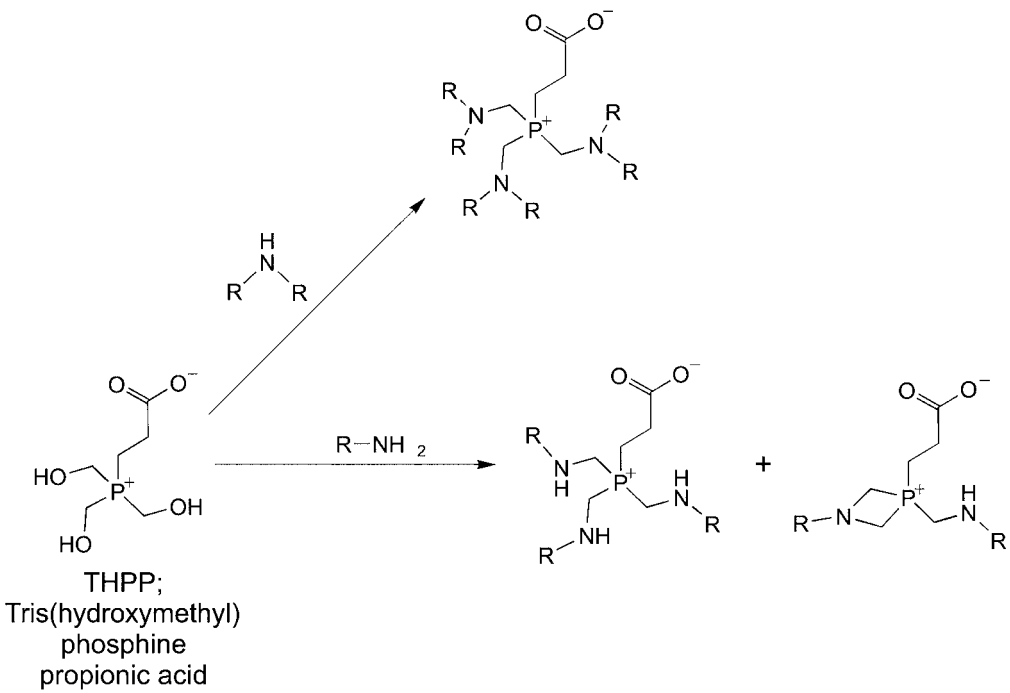


(Reaction 15)

Fluorophenyl ester compounds can be coupled to amines at a pH range of 7–9, with 0.1 M sodium bicarbonate, pH 8, a suggested reaction medium.

1.14. Hydroxymethyl Phosphine Derivatives

Phosphine compounds often are thought of only in terms having reductant properties, especially in biological applications. However, there are classes of phosphine derivatives with hydroxy-methyl group substitutions that also can act as bioconjugation agents for coupling or crosslinking purposes. Tris(hydroxymethyl)phosphine (THP) and β-[tris(hydroxymethyl)phosp hino] propionic acid (THPP; Thermo Fisher) are small trifunctional compounds that spontaneously react with nucleophiles, such as amines, to form covalent linkages (Henderson *et al.*,



(Reaction 16)

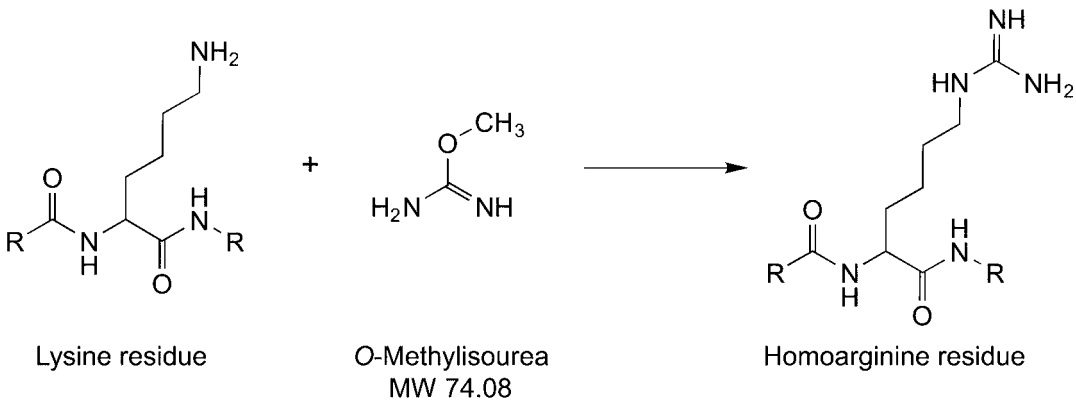
1994; Katti, 1996; Katti *et al.*, 1999). Nucleophiles react with the hydroxymethyl arms by attack on the electron-deficient carbon atom with loss of water to form secondary or tertiary amine bonds (Reaction 16).

Both THP and THPP are stable in aqueous solution, as the only potential product of hydrolysis is the reformation of the hydroxymethyl groups. It is unusual for an amine-reactive functional group to have long-term stability in water or buffer, which makes these reagents uniquely suitable for creating reactive surfaces or reactive molecules for subsequent conjugation with proteins or other amine-containing compounds. Hydroxylic chromatographic supports also have been activated with hydroxymethyl phosphine derivatives for immobilization of enzymes (Petach *et al.*, 1994).

Hydroxymethyl phosphines are susceptible to oxidation to form the phosphine oxide derivative. Therefore, avoid excess oxygen, oxidizing agents, or azide compounds, which react with phosphines in the Staudinger reaction (Chapter 17, Section 5). In addition, metallic surfaces can be modified via the phosphine group to result in hydroxymethyl group substitutions.

1.15. Guanidination of Amines

The addition of a guanidino group to amine-containing molecules can be done using the compound *o*-methylisourea (as the hemisulfate salt). Guanidination has been used to increase the ionization potential of lysine-containing peptides for greater sensitivity in mass spec analysis (Brancia *et al.*, 2000). The process also has been used to add stable isotope labels (^{15}N) to tryptic peptides (Cristea *et al.*, 2004). Upon trypsin digestion, protein samples are cleaved at arginine and lysine residues to yield peptide fragments containing these amino acids at their C-terminal. The guanidine group of arginine is known to aid in the ionization of peptides for MS analysis. The reaction of *o*-methylisourea hemisulfate with lysine ε-amino groups produces homoarginine (Reaction 17), which ionizes far better than lysine and aids in the detection of these peptides (Warwood *et al.*, 2006). The addition of a guanidine group to a lysine residue adds 42.02 Daltons to the resultant modified peptide, which needs to be taken into account for mass spec purposes.



(Reaction 17)

Protocols for guanidination reactions typically use basic conditions to deprotonate all the lysine ε-amino groups, which is necessary to achieve efficient yields. The amount of N-terminal

α-amine labeling is minimal, but may occur to some extent, especially for peptides containing an N-terminal glycine residue (Beardsley and Reilly, 2002). The initial protocols for guanidination usually use reaction times of several hours, but optimization has resulted in decreasing this to 5–10 minutes at elevated temperature. The following protocol is based on the method of Beardsley and Reilly (2002).

Protocol

1. Dissolve 50 mg of *o*-methylisourea hemisulfate in 51 µl of water.
2. Prepare 5 µl of a peptide solution to undergo guanidination at a concentration of 1 pmol/µl and add 5.5 µl of 7 N ammonium hydroxide.
3. Add 1.5 µl of the *o*-methylisourea hemisulfate solution to the peptide solution with mixing.
4. React for 5–10 minutes at 65°C in an oven.
5. Stop the reaction with the addition of 15 µl of 10 percent TFA (v/v).

2. Thiol Reactions

Reactive groups able to couple with sulfhydryl-containing molecules are perhaps the second most common functional groups present on crosslinking or modification reagents. Especially in the design of heterobifunctional crosslinkers, sulfhydryl-reactive groups frequently are present on one of the two ends. The other end of such crosslinkers is often an amine-reactive group that is coupled to a target molecule before the sulfhydryl-reactive end, due to the labile nature of amine acylation chemistries. The primary coupling reactions for modification of sulfhydryls proceed by one of two routes: alkylation or disulfide interchange. Many of the reactive groups that undergo these reactions are stable enough in aqueous environments to allow a two-step conjugation strategy to be used (Chapter 5, Section 1). Once initiated, most of these reactions are rapid and occur in high yield to give stable thioether or disulfide bonds.

2.1. Haloacetyl and Alkyl Halide Derivatives

Three forms of activated halogen derivatives can be used to create sulfhydryl-reactive compounds: haloacetyl (see Chapter 1, Section 5.2), benzyl halides that react through a resonance activation process with the neighboring benzene ring, and alkyl halides that possess the halogen β to a nitrogen or sulfur atom, as in *N*- and *S*-mustards. In each of these compounds, the halogen group is easily displaced by an attacking nucleophilic substance to form an alkylated derivative with loss of HX (where X is the halogen and the hydrogen comes from the nucleophile). Haloacetyl compounds and benzyl halides typically are iodine or bromine derivatives, whereas the halo-mustards mainly employ chlorine and bromine forms (see Chapter 4, Section 10 for examples of homobifunctional reagents that employ reactive halogen groups).

Although the primary utility of active halogen compounds is to modify sulfhydryl groups in proteins or other molecules, the reaction is not totally specific. Iodoacetyl (and bromoacetyl) derivatives can react with a number of functional groups within proteins: the sulfhydryl group of cysteine, both imidazolyl side chain nitrogens of histidine, the thioether of methionine, and

the primary ε-amine group of lysine residues and N-terminal α-amines (Gurd, 1967). The relative rate of reaction with each of these residues is generally dependent on the degree of ionization and thus the pH at which the modification is done. The exception to this rule is methioninyl thioethers which react rapidly at nearly all pH values above 1.7 (Vithayathil and Richards, 1960). The only reaction resulting in one definitive product is that of the alkylation of cysteine sulfhydryls, giving the carboxymethylcysteinyl derivative (Cole *et al.*, 1958) (Reaction 18). Histidine groups may be modified at either nitrogen atom of its imidazolyl side chain, thus producing the possibility of either mono-substituted or di-substituted products (Crestfield *et al.*, 1963). With primary amine groups such as in the side chain of lysine residues, the products of the reaction are either the secondary amine, monocarboxymethyllysine, or the tertiary amine derivative, dicarboxymethyllysine. Methionine thioether groups give the most complicated products, some of which rearrange or decompose unpredictably. The only stable carboxy derivative of methionine is where the terminal methyl group is lost to form carboxymethylhomocysteine, the same product as the reaction of iodoacetate with homocysteine.

(Reaction 18)

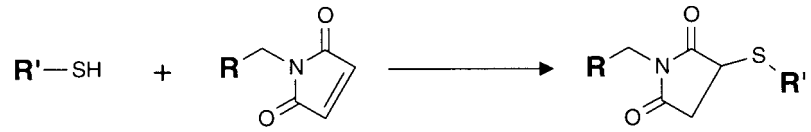
The relative reactivity of α-haloacetates toward protein functional groups is sulfhydryl > imidazolyl > thioether > amine. Among halo derivatives the relative reactivity is I > Br > Cl > F, with fluorine being almost unreactive. The α-haloacetamides have the same trend of relative reactivities, but will create a terminal amide group not a terminal carboxylate.

Thus, iodoacetate has the highest reactivity toward sulfhydryl cysteine residues and may be directed specifically for —SH modification. If iodoacetate is present in limiting quantities (relative to the number of sulfhydryl groups present) and at slightly alkaline pH, cysteine modification will be the exclusive reaction. The specificity of this modification has been used in the design of heterobifunctional crosslinking reagents, where one end of the crosslinker contains an iodoacetamide derivative and the other end contains a different functionality directed at another chemical target (see SIAB, Chapter 5, Section 1.5).

2.2. Maleimides

Maleic acid imides (maleimides) are derivatives of the reaction of maleic anhydride and ammonia or an amine derivative. This functional group is a popular constituent of many heterobifunctional crosslinking agents (Chapter 5). The double bond of maleimides may undergo an alkylation reaction with sulfhydryl groups to form stable thioether bonds. Maleimide reactions are specific for thiols in the pH range of 6.5–7.5 (Smyth *et al.*, 1964; Gorin *et al.*, 1966; Heitz *et al.*, 1968; Partis *et al.*, 1983). At pH 7.0, the reaction of the maleimide with sulfhydryls proceeds at a rate 1000 times greater than its reaction with amines. At higher pH values, some cross-reactivity with amino groups takes place (Brewer and Riehm, 1967). One of the carbons adjacent to the maleimide double bond undergoes nucleophilic attack by the thiolate anion to generate the addition product (Reaction 19). When sufficient quantities of —SH groups

are being alkylated, the reaction may be followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacts and disappears.

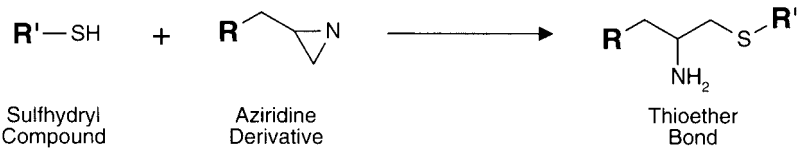


(Reaction 19)

The maleimide group also may undergo hydrolysis to an open maleamic acid form that is unreactive toward sulfhydryls (Chapter 19, Section 5). Hydrolysis may occur after sulfhydryl coupling to the maleimide, as well. This ring-opening reaction typically happens faster the higher the pH becomes. Hydrolysis is also dependent on the type of chemical group next to the maleimide function. For instance, the cyclohexane ring of SMCC (Chapter 5, Section 1.3) provides increased stability to maleimide hydrolysis probably due to its steric effects and its lack of aromatic character. However, the adjacent phenyl ring of MBS allows much greater rates of hydrolysis to occur at the maleimide ring (Chapter 5, Section 1.4).

2.3. Aziridines

An Aziridine reactive group is a small ring system composed of one nitrogen and two carbon atoms. The highly hindered nature of this heterocyclic ring gives it strong reactivity toward nucleophiles. Sulfhydryls will react with aziridine-containing reagents in a ring-opening process, forming thioether bonds (Reaction 20). The simplest aziridine compound, ethylenimine, can be used to transform available sulfhydryl groups into amines (Chapter 1, Section 4.3).



(Reaction 20)

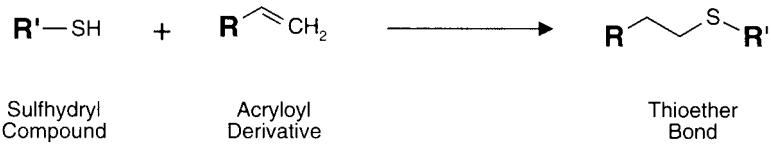
The reaction of an aziridine with a thiol is highly specific at slightly alkaline pH values. In aqueous solution, the major side reaction is hydrolysis.

Substituted aziridines have been used to form homobifunctional and trifunctional crosslinking agents, although their use has been limited (Ross, 1953; Alexander, 1954). The functional group has found use, however, in the design of the fluorescent probe dansyl aziridine (5-dimethylaminonaphthalene-2-sulfonyl aziridine) (Johnson *et al.*, 1978; Grossman *et al.*, 1981).

2.4. Acryloyl Derivatives

Reactive double bonds are capable of undergoing addition reactions with sulfhydryl groups. A popular example of this type of functional group is the maleimide group (Section 2.2, this

chapter). However, derivatives of acrylic acid also are able to participate in this reaction, although the rate of sulfhydryl addition is somewhat slower than that of maleimides. The reaction of an acryloyl compound with a sulfhydryl group occurs with the creation of a stable thioether bond (Reaction 21).



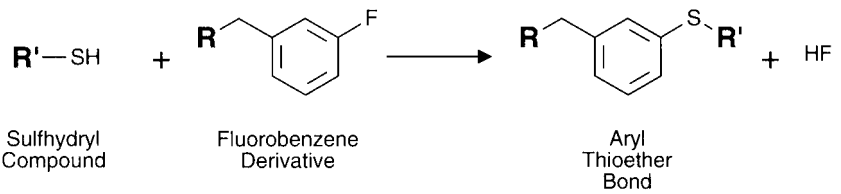
(Reaction 21)

Although acryloyl crosslinking agents have not been common, the reactive group has found use in the design of the sulfhydryl-reactive fluorescent probe, 6-acryloyl-2-dimethylaminonaphthalene (acrylodan; Molecular Probes) (Epps *et al.*, 1992; Yem *et al.*, 1992).

2.5. Arylating Agents

Arylating agents are reactive aromatic compounds containing a constituent on the ring that can undergo nucleophilic substitution. The most common arylating agents are derivatives of benzene which possess either halogen or sulfonate groups on the ring. The presence of electron withdrawing constituents, such as nitro groups, increases the reactivity of the replaceable group. Although aryl halides are commonly used to modify amine-containing molecules to form aryl amine derivatives, they also react quite readily with sulfhydryl groups.

Fluorobenzene-type compounds have been used as functional groups in homobifunctional crosslinking agents (Chapter 4, Section 4). Their reaction with nucleophiles involves bimolecular nucleophilic substitution, causing the replacement of the fluorine atom with the sulfhydryl derivative, and creating a substituted aryl bond (Reaction 22). Conjugates formed with sulfhydryl groups are reversible by cleaving with an excess of thiol (such as DTT) (Shaltiel, 1967). Detection reagents incorporating reactive aryl chemistry include 2,4-dinitrofluorobenzene and trinitrobenzenesulfonate (Eisen *et al.*, 1953). The relative rate of reactivity for aryl compounds is: F > Cl ~ Br > Sulfonate.

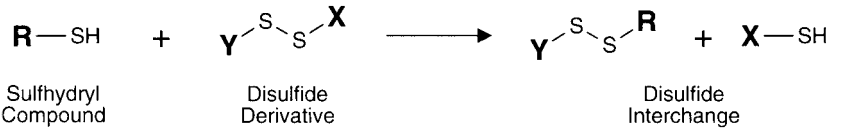


(Reaction 22)

2.6. Thiol-Disulfide Exchange Reagents

Compounds containing a disulfide group are able to participate in disulfide exchange reactions with another thiol. The disulfide exchange (also called interchange) process involves attack of the thiol at the disulfide, breaking the —S—S— bond, with subsequent formation of a new mixed disulfide comprising a portion of the original disulfide compound (Reaction 23). The reduction

of disulfide groups to sulfhydryls in proteins using thiol-containing reductants proceeds through the intermediate formation of a mixed disulfide (Chapter 1, Section 4.1). If the thiol is present in excess, the mixed disulfide can go on to form a symmetrical disulfide consisting entirely of the thiol reducing agent—thus completely reducing the original disulfide to free sulfhydryls. If the thiol reductant is not present in large enough excess, the mixed disulfide product is the end result.



(Reaction 23)

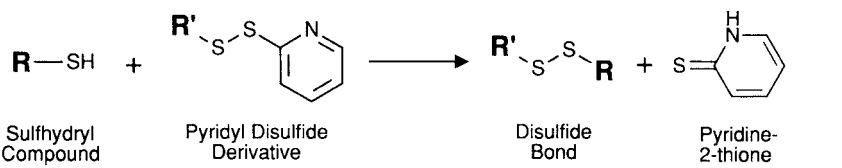
Crosslinking or modification reactions using disulfide exchange processes form disulfide linkages with sulfhydryl-containing molecules. These bonds are reversible using disulfide reducing agents. Thus, conjugates may be created and latter released for analysis by incubation with DTT or other disulfide reductants (e.g., TCEP). The disulfide bond within these crosslinks also permits important reactions to occur *in vivo*, such as the release of the toxin component of immunotoxin conjugates, allowing the cytotoxic portion to penetrate target cells and cause cell death (Chapter 21).

Disulfide exchange reactions occur over a broad range of conditions—from acid to basic pH—and in a wide variety of buffer constituents. Most crosslinking reactions involving disulfide exchange are done under physiological conditions or those most appropriate to maintain stability of the protein or other molecule being modified.

Pyridyl Disulfides

A pyridyl dithiol is perhaps the most popular type of thiol-disulfide exchange functional group used in the construction of crosslinkers or modification reagents. Pyridyl disulfides can be created from available primary amines on molecules through the reaction of 2-iminothiolane in tandem with 4,4′-dipyridyl disulfide (King *et al.*, 1978). For instance, the simultaneous reaction between a protein, 2-iminothiolane, and 4,4′-dipyridyl disulfide yields a modification containing reactive pyridyl disulfide groups in a single step (Chapter 1, Section 4.1).

A pyridyl disulfide will readily undergo an interchange reaction with a free sulfhydryl to yield a single mixed disulfide product. This is due to the fact that the pyridyl disulfide contains a leaving group that is easily transformed into a non-reactive compound not capable of participating in further mixed disulfide formation. Thus, the thiol-disulfide exchange reaction can be controlled to occur with only one-half of the original disulfide compound. For instance, a reagent system containing a pyridyl disulfide group, such as SPDP (Chapter 5, Section 1.1), is able to react with sulfhydryl groups by releasing the electron-stabilized compound, pyridine-2-thione (Reaction 24). Since the leaving group does not possess a free thiol, it can not disulfide exchange with another molecule of the attacking sulfhydryl compound. Thus, only one end of the reagent has potential for becoming attached to the sulfhydryl-containing molecule.

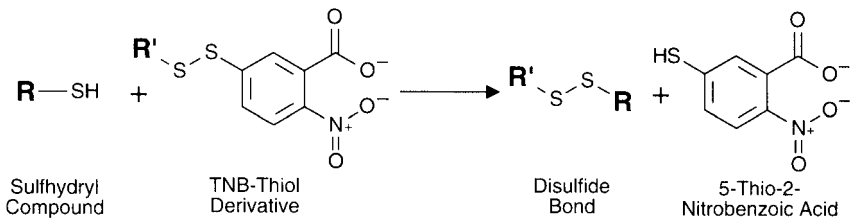


(Reaction 24)

Pyridyl dithiol containing crosslinking and modification reagents are highly efficient in forming disulfide bonds with sulfhydryl-containing molecules. In addition, the pyridine-2-thione leaving group has unique spectral properties that allow the measurement of sulfhydryl coupling by monitoring the increase in absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3/M^{-1}cm^{-1}$). Once a disulfide linkage is formed, it may be cleaved using standard disulfide reducing agents (Chapter 1, Section 4.1).

TNB-Thiol

Sulfhydryl groups activated with the leaving group 5-thio-2-nitrobenzoic acid can be used to couple free thiols by disulfide interchange similar to pyridyl disulfides, as discussed previously. A TNB-thiol-activated species may be created by reaction of a sulfhydryl group with Ellman's reagent, 5,5′-dithio-*bis*(2-nitrobenzoic acid) or DTNB, a compound useful for the quantitative determination of sulfhydryls in solution (Ellman, 1958, 1959) (Chapter 1, Section 4.1). The disulfide of Ellman's reagent readily undergoes disulfide exchange with a free sulfhydryl to form a mixed disulfide with concomitant release of one molecule of the chromogenic substance 5-sulfido-2-nitrobenzoate, also called 5-thio-2-nitrobenzoic acid (TNB). The TNB-thiol group can again undergo interchange with a sulfhydryl-containing target molecule to yield a disulfide crosslink. Upon coupling with a sulfhydryl compound, the TNB group is released (Reaction 25). The intense yellow color produced by the TNB anion can be measured by its absorbance at 412 nm ($\varepsilon = 1.36 \times 10^4/M^{-1}cm^{-1}$ at pH 8.0). Since each sulfhydryl which is coupled generates one molecule of TNB per molecule of Ellman's reagent, the possibility for quantifying the reaction exists.

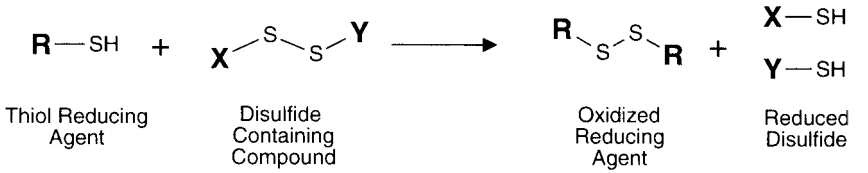


(Reaction 25)

Disulfide exchange with a TNB-thiol group occurs efficiently at physiological to slightly alkaline pH conditions. Avoid the presence of disulfide reducing agents, as these will cleave the TNB group and prevent specific coupling.

Disulfide Reductants

Disulfide reduction by the use of disulfide interchange can be done using thiol-containing compounds such as TCEP, DTT, 2-mercaptoethanol, or 2-mercaptoethylamine (Chapter 1, Section 4.1). The formation of free sulfhydryls from a disulfide group occurs in two stages. First, one molecule of the reducing agent undergoes disulfide exchange, cleaving the disulfide and forming a new, mixed disulfide. In the next stage, a second molecule of the thiol cleaves the mixed disulfide, releasing a free sulfhydryl and forming a molecule of oxidized reducing agent (Reaction 26).

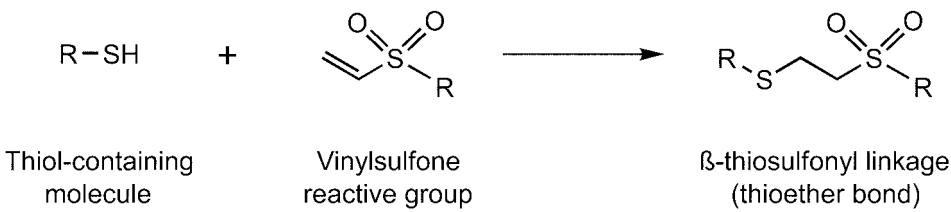


(Reaction 26)

Disulfide reduction occurs over a broad pH range and in a variety of buffer environments. The reaction can be done in denaturants, chaotropic agents, detergents, and in high salt conditions.

2.7. Vinylsulfone Derivatives

A vinylsulfone group can be used to conjugate with nucleophiles, especially thiol groups, in aqueous solution and under mild conditions (Masri and Friedman, 1988). In addition to thiols, they can react with amines and hydroxyls under higher pH conditions. As opposed to a maleimide group, the vinylsulfone is not as strong an electrophile, but efficiently couples with thiols at slightly alkaline pH values to give stable β-thiosulfonyl linkages (Reaction 27). The product of the reaction of a thiol with a vinylsulfone gives a single stereoisomer structure, unlike conjugation with maleimides, which produces two potential stereoisomers. In addition, crosslinkers and modification reagents containing a vinylsulfone can be used to activate surfaces or molecules to contain thiol-reactive groups. These vinylsulfone groups are stable in aqueous solution for extended periods, as they are not subject to hydrolysis at neutral pH. Thus, they retain excellent coupling potential for thiol-containing proteins or other molecules even when used in aqueous buffer conditions.



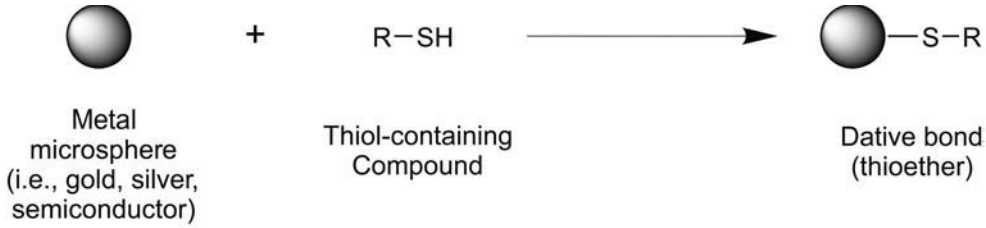
(Reaction 27)

Vinylsulfone activated chromatography supports long have been used for coupling affinity ligands that contain thiols or other nucleophiles (Porath, 1974). The reactive group also has been used to activate PEG polymers for modification of thiol-containing molecules (Morpurgo *et al.*, 1996). There now are homobifunctional and heterobifunctional crosslinking agents commercially available that use the vinylsulfone reactive group (Thermo Fisher and Molecular Biosciences).

2.8. Metal-Thiol Dative Bonds

Thiol-containing molecules can interact with metal ions and metal surfaces to form dative bonds. Dative bonds also are known as coordinate covalent bonds. They differ from normal

covalent linkages, because they are formed by two electrons coming from a single atom, instead of two atoms each sharing one electron. In a coordinate bond formed with a thiol, the unshared pair of electrons on the sulfur atom is able to form a dative bond with a metal atom. In this sense, even disulfides are able to datively link to a metal surface without prior reduction to thiols (Reaction 28).



(Reaction 28)

Other atoms containing a lone pair of electrons also are effective at forming coordinate bonds. Oxygen- and nitrogen-containing organic molecules often are used to chelate metal ions, such as in various lanthanide chelates (Chapter 9, Section 9), bifunctional metal-chelating compounds (Chapter 10), and FeBABE (Chapter 28, Section 4.1). In addition, amino acid side chains and prosthetic groups in proteins frequently form bioinorganic motifs by coordinating a metal ion as part of an active center (Degtyarenko, 2000). Thiol organic compounds, however, are used routinely to coat metallic surfaces or particles to form biocompatible layers or create functional groups for further conjugation of biomolecules.

Thiol-ligand modification in particular has been used extensively to create water soluble quantum dots (Sapsford *et al.*, 2006) and gold nanoparticles having reduced nonspecific binding character to the metallic surface and to form surface groups for coupling proteins and other affinity molecules (Chapter 9, Section 10 and Chapter 24). For instance, thiol-containing aliphatic/PEG linkers have been used to form self-assembled monolayers (SAMs) on planar gold surfaces and particles (Prime and Whitesides, 1991).

A monodentate thiol-metal bond is not as strong as a true covalent linkage. These bonds are subject to displacement by other molecules containing thiols or other atoms with a lone pair of electrons. The thiol also may oxidize off the surface if exposed to oxygen in air or aqueous solutions. Instead of monodentate compounds, the use of multidentate molecules can dramatically increase the stability of a coordination bond. A bidentate DOPA ligand, for instance, was found to have far greater bonding adhesion to a surface than monodentate ligands and nearly as much as a covalent bond (Lee *et al.*, 2006).

To increase the strength of thiol dative linkages, the application of multivalent thiols has been used to increase the overall strength of a single molecule tether bound to a metal. Bidentate thiol ligands that have been designed for metal surface modification include lipoic acid (thioctic acid) derivatives (Cheng and Brajter-Toth, 1992; Willey *et al.*, 2004; Hahn *et al.*, 2007), dithiobis(succinimidyl)propionate (DSP) modifications (Grubor *et al.*, 2004) (Chapter 4, Section 1.1), and a dithiol linker built from a central phenyl ring, which contains a PEG spacer for hydrophilicity on the SAM surface (Spangler *et al.*, 2004) (Figures 2.2 and 2.3).

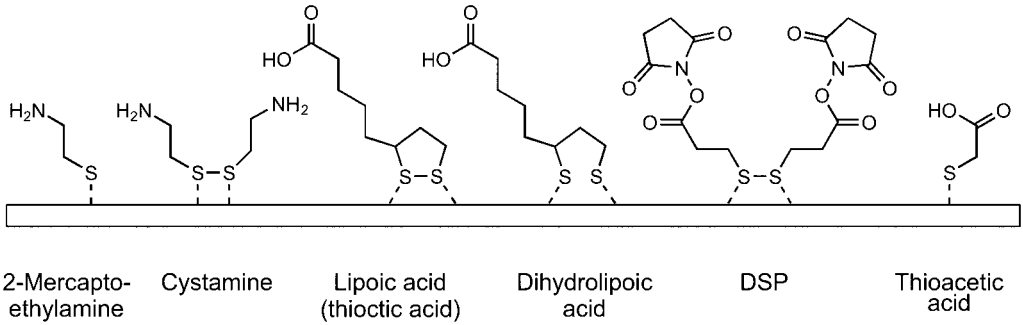


Figure 2.2 A number of small thiol-containing molecules have proven useful for modification of gold or metallic surfaces. The dithiol derivatives provide better dative bond stability and can't be displaced easily by competing thiols or oxidation. Most thiol-containing compounds used for surface modification also contain terminal functional groups or reactive groups for coupling affinity ligands.

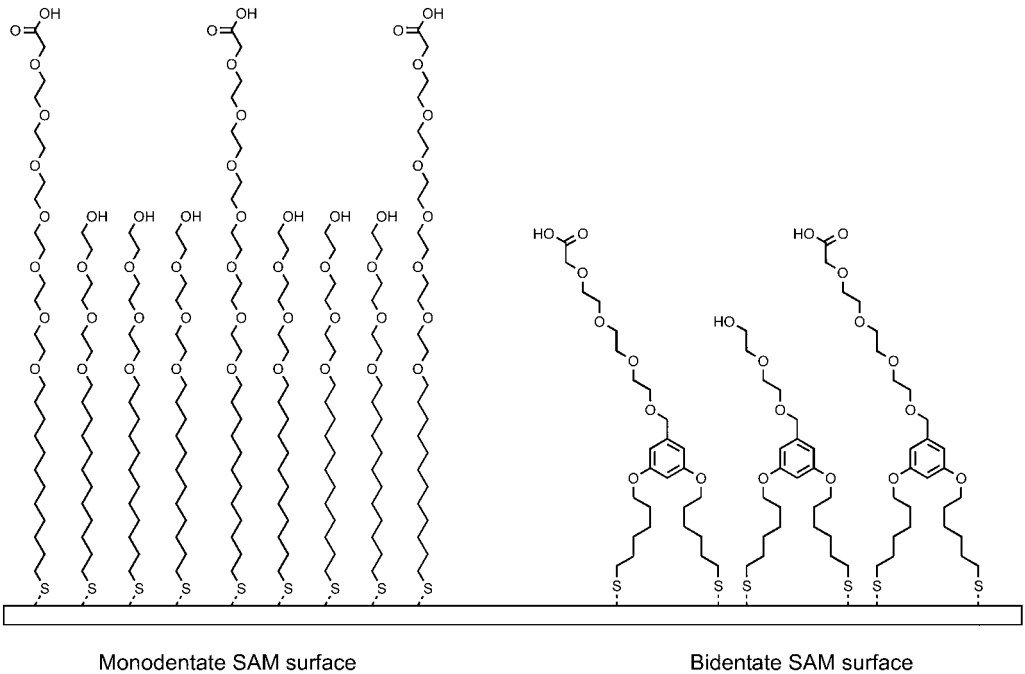
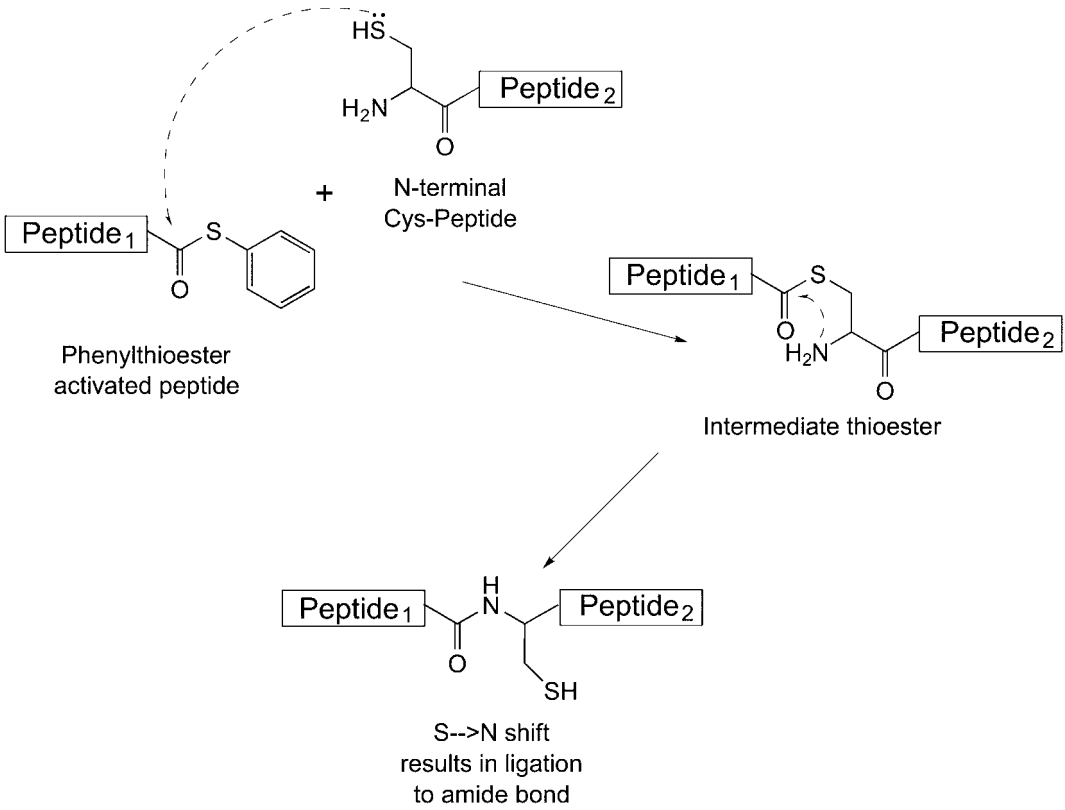


Figure 2.3 SAM surface modification has been done using monothiol and dithiol compounds containing PEG linkers. Useful coatings typically contain mainly PEG-hydroxyl or PEG-monomethyl ether linkers that provide a biocompatible lawn, which prevents nonspecific binding of proteins to the metallic surface. About 10 percent of the surface modifications are done using a longer carboxylate-containing thiol-PEG linker that provides sites for attachment of affinity ligands.

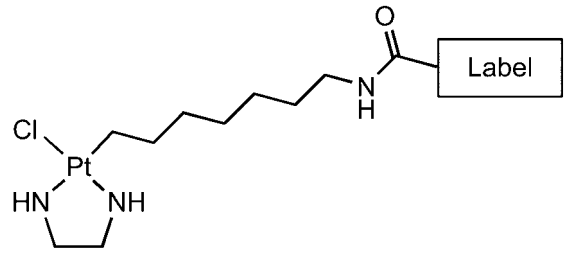
2.9. Native Chemical Ligation

Native chemical ligation involves reactions that are very similar to intein splicing and peptide ligation found in certain protein expression systems. A peptide having a C-terminal thioester reacts with an N-terminal cysteine residue in another peptide to undergo a transthioesterification reaction, which results in the formation of an intermediate thioester with the cysteine thiol. However, due to the proximity of the neighboring α-amine group on cysteine, a subsequent nucleophilic attack of the electron-rich nitrogen on the ester carbonyl results in an S→N shift, this then forms a native amide (peptide) bond (Reaction 29).



(Reaction 29)

Native chemical ligation is effective at conjugating two peptides together in aqueous solution to form a longer peptide. The reaction proceeds at physiological pH under mild conditions without any additional additives, except for the presence of the thioester-containing peptide and the cysteine-containing peptide. In addition, other thioester compounds may be used in this reaction to label specifically N-terminal cysteine peptides through amide bond formation. For instance, a biotin thioester derivative may be used to add a biotin group to a peptide or a fluorescent label may be added by the same process. Thus, native chemical ligation is an excellent way of discretely labeling peptides only at their N-terminal. See Chapter 17, Section 6 for additional information on the use of this reaction.



Cisplatin modification agent
MW 444.86
(without label)

Figure 2.4 The general design of a cisplatin modification agent consists of the reactive cisplatin group and a short linker that typically terminates in a detectable label.

2.10. Cisplatin Modification of Methionine and Cysteine

Platinum complexes long have been used as tumor therapeutic agents for their ability to bind to DNA at guanine and adenine residues and interfere with transcription (Repta and Long, 1980; Eastman, A., 1987; Reedijk *et al.*, 1987). The reaction of Platinum II compounds with nucleic acids has been studied in great detail (Anin *et al.*, 1992), and the reactive group has been applied to bioconjugation labeling reagents (van Belkum *et al.*, 1994; Heetebrij *et al.*, 2003).

Cisplatin reagents also react with certain amino acid residues in proteins. Rapid coupling occurs with methionine and cysteine residues over a broad pH range (2–9), while slower reaction kinetics results in modification of histidine imidazole ring with an optimal pH at 8.0. The relative rate of reaction of cisplatin derivatives for sulfur nucleophiles is over 100 times more than their reaction with nitrogen nucleophiles (Hay and Porter, 1999). The basic structure of cisplatin labeling reagents is shown in Figure 2.4 and Figure 2.5 illustrates the modification reactions of a cisplatin derivative with amino acid or nucleic acid targets. The reactive group is stable in aqueous conditions and covalent bonds formed with it are stable to typical conditions used in biological assays and detection procedures. Reagent systems are available from Kreatech.

3. Carboxylate Reactions

Chemical groups that specifically react with carboxylic acids are limited in variety. In aqueous solutions, the carboxylate functionality displays rather low nucleophilicity. For this reason, it is unreactive with the great majority of bioconjugate reagents which couple through a nucleophilic addition process.

Several important chemistries, however, have been developed that allow conjugation through a carboxylate group. The following sections briefly describe these reactions.

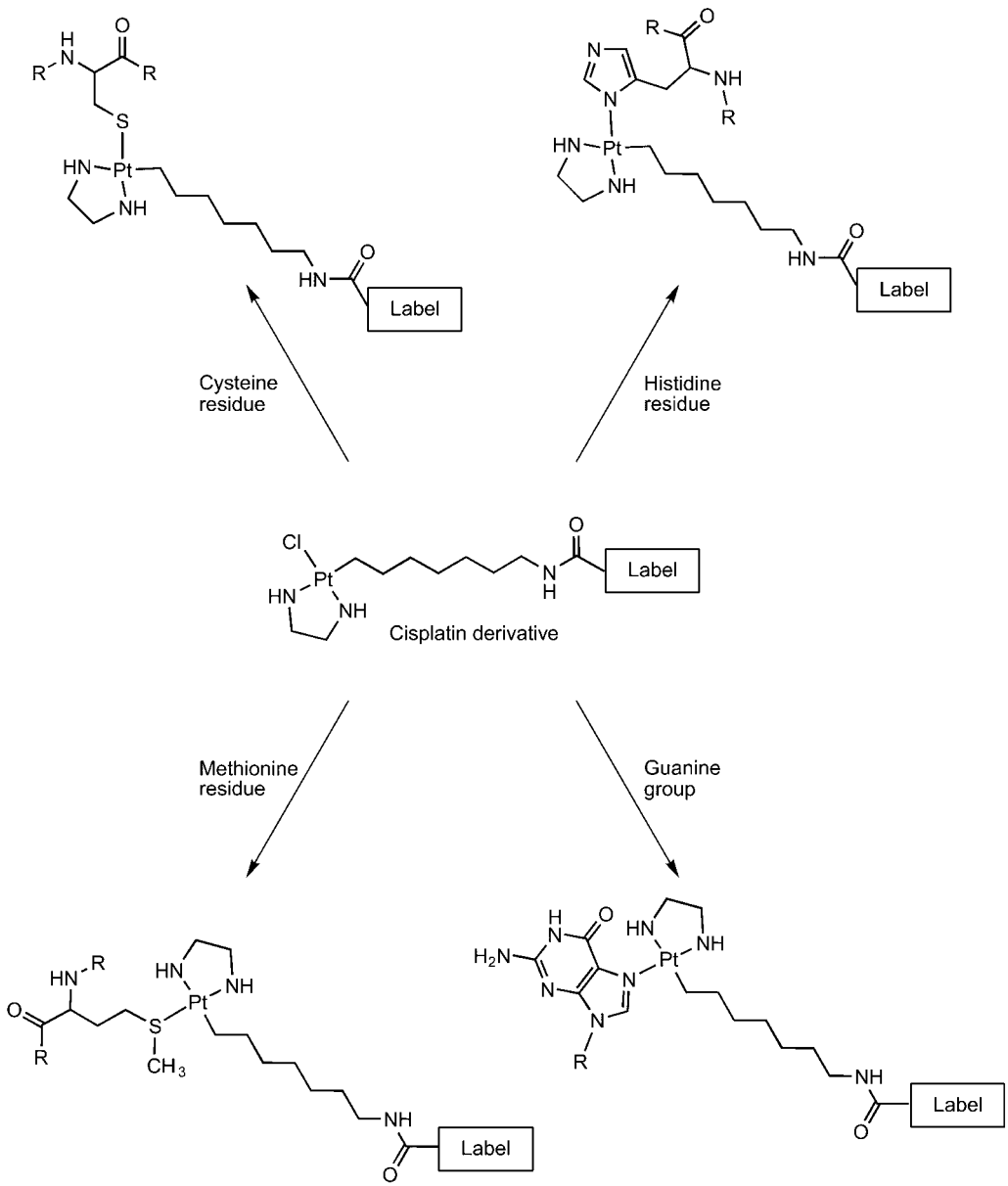


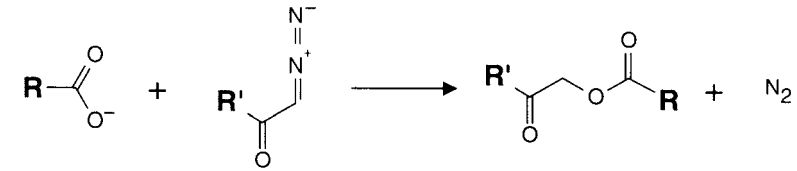
Figure 2.5 The cisplatin reactive group can covalently couple to methionine-, cysteine-, and histidine-containing peptides or proteins. It also reacts with guanine groups to form a covalent modification on the N_7 nitrogen.

3.1. Diazoalkanes and Diazoacetyl Compounds

Diazomethane and other diazoalkyl derivatives long have been used to label carboxylate groups for analysis (Herriott, 1947; Riehm and Scheraga, 1965). A major application of such

reagents has been in the HPLC analysis of low-molecular-weight compounds such as fatty acids (DeMar *et al.*, 1992). Several coumarin derivatives containing stable, carboxylate-reactive diazoalkane functionalities also are available for fluorescent-labeling of target molecules (Molecular Probes) (Ito and Sawanobori, 1982; Ito and Maruyama, 1983).

Diazoalkanes and diazoacetyl compounds (amides and esters) are spontaneously reactive with carboxylate groups without addition of other reactants or catalysts. The reaction mechanism involves attack of a negatively charged carboxylate oxygen atom on a protonated diazoalkyl group, liberating nitrogen gas, and forming a covalent linkage (Reaction 30).

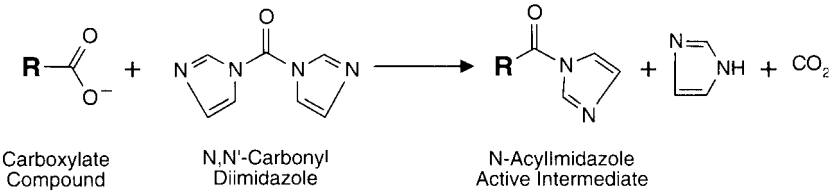


(Reaction 30)

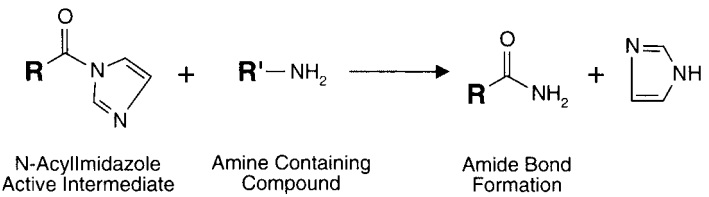
The reaction with carboxylates occurs over a range of pH values, but is optimal at pH 5.0. Unfortunately, the diazoalkyl compounds will cross-react with sulfhydryl groups at this pH. At higher pH conditions, the reaction is even less specific due to reaction with other nucleophiles. In aqueous solution, the most-likely side reaction is hydrolysis.

3.2. Carbonyldiimidazole

CDI, is a active carbonylating agent that contains two acylimidazole leaving groups (Chapter 3, Section 3). CDI reacts with carboxylic acids under non-aqueous conditions to form *N*-acylimidazoles of high reactivity (Reaction 31). The active intermediate forms in excellent yield due to the driving force created by the liberation of carbon dioxide and imidazole (Anderson, 1958). An active carboxylate then can react with amines to form amide bonds or with hydroxyl groups to form ester linkages (Reaction 32). The reaction has been used successfully in peptide synthesis (Paul and Anderson, 1960, 1962). In addition, activation of a styrene/4-vinylbenzoic acid copolymer with CDI was used to immobilize the enzyme lysozyme through its available amino groups to the carboxyl groups on the matrix (Bartling *et al.*, 1973).



(Reaction 31)



(Reaction 32)

CDI functions as a zero-length crosslinker if the activated species is a carboxylic acid, because the attack of another nucleophile liberates the imidazole leaving group. The conjugation reaction can be done in organic solvent or aqueous conditions, depending on the solubility of the nucleophile. For aqueous coupling of *N*-acylimidazoles to amine-containing compounds, optimal conditions include an alkaline pH environment from about pH 7–9 and in buffers containing no amines (avoid Tris or imidazole).

3.3. Carbodiimides

Carbodiimides function as zero-length crosslinking agents capable of activating a carboxylate group for coupling with an amine-containing compound. There are several major types of carbodiimide reagents commonly available that can be used for organic or aqueous reactions, depending on their individual solubility characteristics (Chapter 3, Section 1). The water soluble reagents are used mainly for biological conjugations involving proteins and other macromolecules. The water-insoluble carbodiimides can be used in peptide synthesis or for the synthesis of other organic compounds.

Carbodiimides are used to mediate the formation of amide or phosphoramidate linkages between a carboxylate and an amine or a phosphate and an amine, respectively (Hoare and Koshland, 1966; Chu *et al.*, 1986; Ghosh *et al.*, 1990). Regardless of the type of carbodiimide, the reaction proceeds by the formation of an intermediate *o*-acylisourea that is highly reactive and short-lived in aqueous environments. The attack of an amine nucleophile on the carbonyl group of this ester results in the loss an isourea derivative and formation of an amide bond (see Reactions 11 and 12). The major competing reaction in water is hydrolysis.

4. Hydroxyl Reactions

Hydroxyl-reactive chemical compounds include not only those modification agents able to directly form a stable linkage with an —OH group, but also a broad range of reagents that are designed to temporarily activate the group for coupling with a secondary functional group. Many of the chemical methods for modifying hydroxyls originally were developed for use with chromatography supports in the coupling of affinity ligands. Some of these same chemical reactions have found application in bioconjugate techniques for crosslinking a hydroxyl-containing molecule with another substance, usually containing a nucleophile. For instance, carbohydrate-containing molecules such as polysaccharides or glycoproteins can be coupled through their sugar residues using hydroxyl-specific reactions. In addition, polymers and other organic compounds containing hydroxyls (such as PEG) may be conjugated with another molecule using these chemistries.

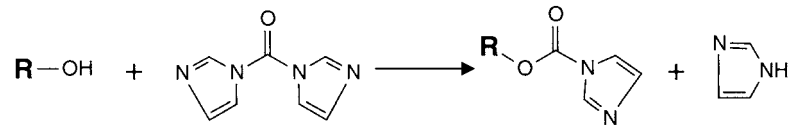
4.1. Epoxides and Oxiranes

An epoxide or oxirane group can react with nucleophiles in a ring-opening process. The reaction can take place with primary amines, sulfhydryls, or hydroxyl groups to create secondary

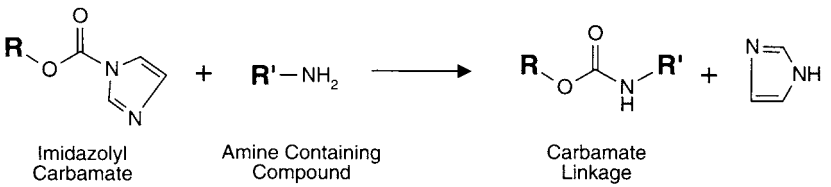
amine, thioether, or ether bonds, respectively. See Section 1.7 (this chapter) for further information on this reaction.

4.2. Carbonyldiimidazole

CDI, is a active carbonylating agent that contains two acylimidazole leaving groups (Chapter 3, Section 3). The compound can react with a carboxylate to form an active acylimidazole group capable of coupling with amine-containing molecules (Section 3.2, this chapter). However, CDI also can react with hydroxyl groups to create a reactive intermediate. If CDI is used to activate a hydroxyl functional group, the reaction proceeds quite differently from its reaction with carboxylates. The active intermediate formed by the reaction of CDI with an —OH group is an imidazolyl carbamate (Reaction 33). Attack by an amine releases the imidazole, but not the carbonyl. Thus, hydroxyl-containing molecules may be coupled to amine-containing molecules with the result of a one-carbon spacer, forming stable urethane (*N*-alkyl carbamate) linkages (Reaction 34). This coupling procedure has been applied to the activation of hydroxyl-containing chromatography supports for the immobilization of amine containing affinity ligands (Bethell *et al.*, 1979; Hearn *et al.*, 1979, Hearn *et al.*, 1983) and also to the activation of PEG for the modification of amine-containing macromolecules (Beauchamp *et al.*, 1983).



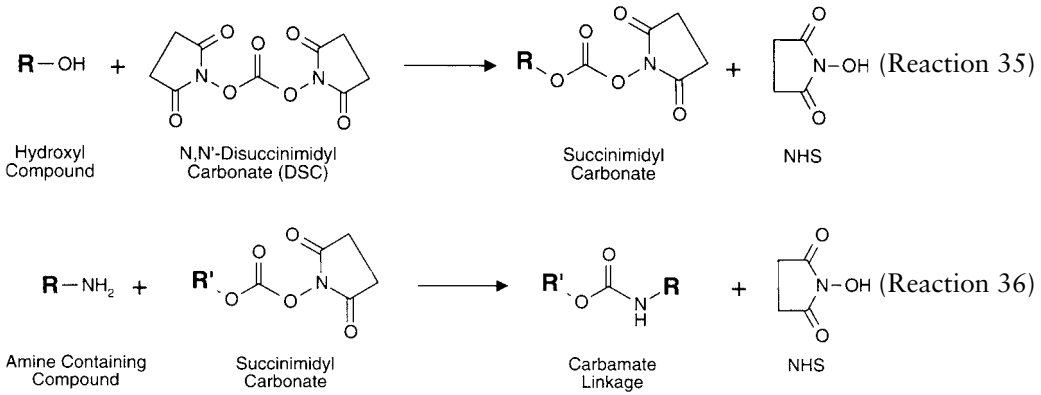
(Reaction 33)



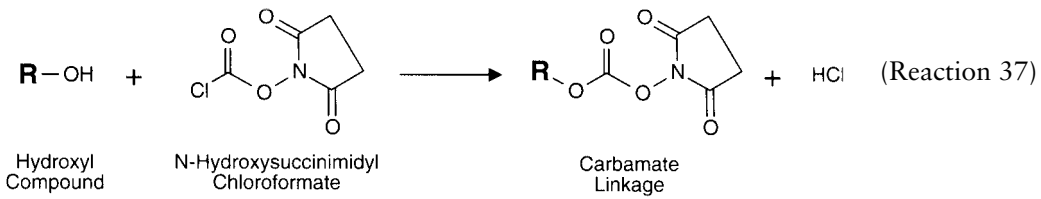
(Reaction 34)

4.3. *N,N′*-Disuccinimidyl Carbonate or *N*-Hydroxysuccinimidyl Chloroformate

N,N′-Disuccinimidyl carbonate (DSC) consists of a carbonyl group containing, in essence, two NHS esters. The compound is highly reactive toward nucleophiles. In aqueous solutions, DSC will hydrolyze to form two molecules of NHS with release of one molecule of CO_2. In non-aqueous environments, the reagent can be used to activate a hydroxyl group to a succinimidyl carbonate derivative (Reaction 35). DSC activated hydroxylic compounds can be used to conjugate with amine-containing molecules to form stable crosslinked products (Reaction 36). The linkage created from this reaction is a urethane derivative or a carbamate bond, displaying excellent stability.

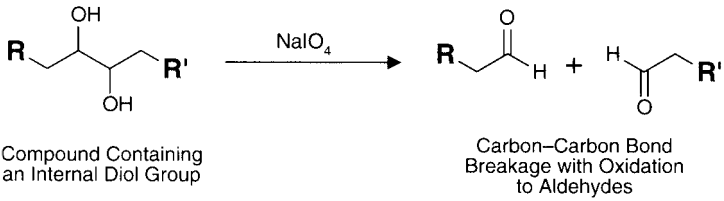


A related reagent, *N*-hydroxysuccinimidyl chloroformate also is a bifunctional carbonyl derivative containing an NHS ester and a acid chloride. In aqueous solutions, the compound is unstable to hydrolysis, rapidly breaking down to NHS, CO_2, and HCl. In non-aqueous environments, however, NHS-chloroformate may be used to activate a hydroxyl group similar to DSC. Reaction of the chloroformate with a hydroxylic residue forms the same succinimidyl carbonate derivative as the reaction of DSC with —OH groups (Reaction 37). Subsequent conjugation with an amine-containing compound yields a carbamate linkage. The bond is identical to that formed from the reaction of CDI activated hydroxyls with amine-containing compounds (see previous section).



4.4. Oxidation with Periodate

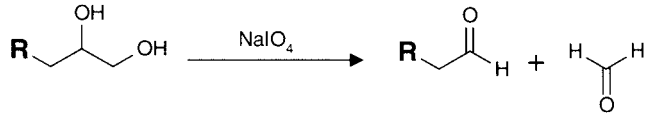
Sodium periodate can be used to oxidize hydroxyl groups on adjacent carbon atoms, forming reactive aldehyde residues suitable for coupling with amine or hydrazide-containing molecules. The reaction occurs with two adjacent secondary hydroxyls to cleave the carbon–carbon bond between them and create two terminal aldehyde groups (Reaction 38). When one of the adjacent hydroxyls is a primary hydroxyl, reaction with periodate releases one molecule of formaldehyde and leaves a terminal aldehyde residue on the original diol compound (Reaction 39). These reactions can be used to generate crosslinking sites in carbohydrates or glycoproteins for subsequent conjugation of amine-containing molecules by reductive amination (Chapter 1, Section 4.4 and Chapter 3, Section 4). Sodium periodate also reacts with 2-aminoethanol derivatives—compounds containing a primary amine and a secondary hydroxyl group on adjacent carbon atoms. Oxidation cleaves the carbon–carbon bond, forming a terminal aldehyde group on the side that had the original hydroxyl residue (Reaction 40). This reaction can be used to create reactive aldehydes on N-terminal serine residues of peptides (Geoghegan and Stroh, 1992).



Compound Containing an Internal Diol Group

Carbon–Carbon Bond Breakage with Oxidation to Aldehydes

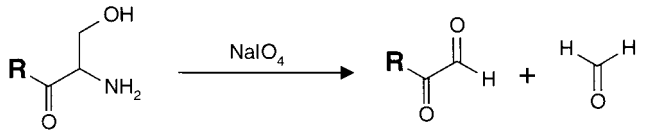
(Reaction 38)



Compound Containing Terminal Diol Group

Oxidation to Aldehyde with Release of Formaldehyde

(Reaction 39)



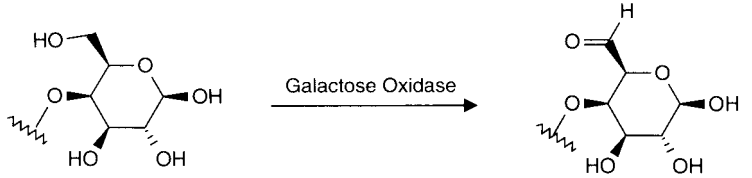
Compound Containing Terminal Hydroxylamine Group

Oxidation to Aldehyde with Release of Formaldehyde

(Reaction 40)

4.5. Enzymatic Oxidation

Certain enzymes may be used to oxidize hydroxyl-containing carbohydrates to create aldehyde groups (Chapter 1, Section 4.4). For example, the reaction of galactose oxidase on terminal galactose or *N*-acetyl-D-galactose residues proceeds to form C-6 aldehyde groups on polysaccharide chains (Reaction 41). These groups then can be used for conjugation reactions with amine or hydrazide-containing molecules.



ß-D-Galactose Residue at the end of a Polysaccharide Chain

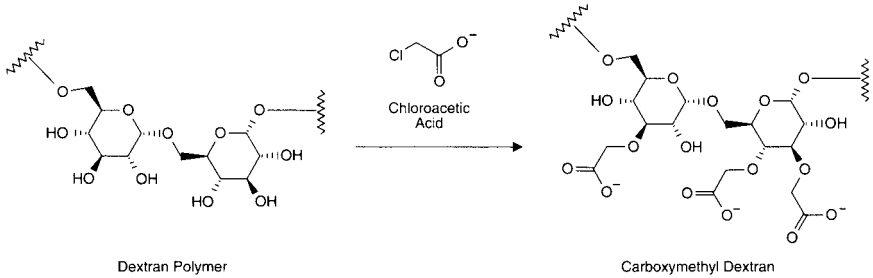
Selective Oxidation of only Terminal Residue (or N-acetyl-galactosamine)

(Reaction 41)

4.6. Alkyl Halogens

Reactive alkyl halogen compounds can be used to modify specifically hydroxyl groups in carbohydrates, polymers, and other molecules. Chloro- or bromo-derivatives of short alkyl chains

containing an electron withdrawing second functional group on their other end (typically a carboxylate group) can be used to form spacer arms useful for conjugation with another substance. Brunswick *et al.* (1988) used chloroacetic acid to modify the hydroxyl groups of dextran, forming the carboxymethyl derivative (Reaction 42). The carboxylates then may be coupled with amine-containing molecules using a carbodiimide reaction scheme. In a somewhat similar approach, Noguchi *et al.* (1992) prepared a carboxylate spacer arm by reacting 6-bromohexanoic acid with a dextran polymer (Chapter 25, Section 2.2).

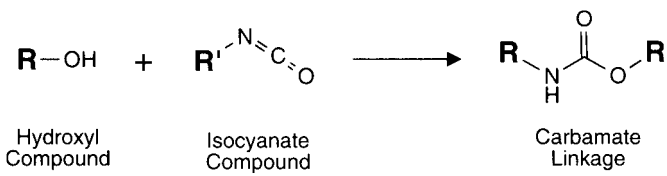


(Reaction 42)

Modification of hydroxyl groups with such compounds can be done in 3–10 M NaOH by reacting from 25°C to 40°C for 1.5–4 hours.

4.7. Isocyanates

Isocyanates can be formed from the reaction of an aromatic amine with phosgene (Rifai and Wong, 1986). They also can be created from acyl azides by treatment at 80°C in the presence of an alcohol (Chapter 9, Section 5). In the transformation, the acyl azide group rearranges to form an isocyanate that can react with hydroxyl-containing molecules to form a urethane (carbamate) linkage (Reaction 43). The reactivity of isocyanates is excellent, but for the same reason their stability can be a problem. In storage, moisture decomposes them, releasing CO_2 and leaving an aromatic amine in its place. In aqueous environments, the aromatic amine can react with another molecule of isocyanate to form a urea derivative (Annunziato *et al.*, 1993).



(Reaction 43)

Isocyanate-containing reagents can be used to crosslink or label hydroxyl-containing molecules, including polysaccharides. Carbohydrate modification can be done without the need for prior oxidation of sugar residues with periodate to form reactive aldehydes, as is common in many protocols (Chapter 1, Section 4.4). The reaction occurs best at alkaline pH values (e.g., pH 8.5). Many coupling protocols avoid the hydrolysis problem by performing the reaction in organic solvent (i.e., DMSO).

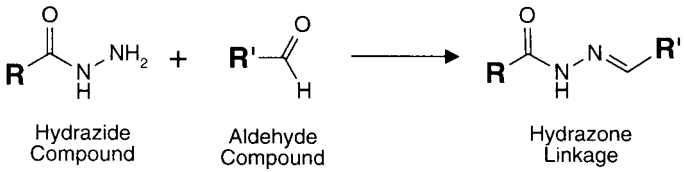
Annunziato *et al.* (1993) have reported on the synthesis and use of a novel heterobifunctional crosslinking reagent containing a hydroxyl-reactive isocyanate group on one end and a sulfhydryl-reactive maleimide group on the other end. The compound can be useful in labeling hydroxylic molecules for subsequent conjugation with thiol-containing molecules.

5. Aldehyde and Ketone Reactions

Aldehyde and ketone groups are important reactive sites in molecules for many bioconjugate strategies. Although some pharmacological agents contain ketones, these groups usually are not present in proteins and other biological macromolecules. Even when a molecule does not contain these functionalities, however, they may be created through a number of processes (Chapter 1, Section 4.4). The following sections discuss the major reactions that can be done with aldehydes and ketones to modify or crosslink molecules containing them.

5.1. Hydrazine Derivatives

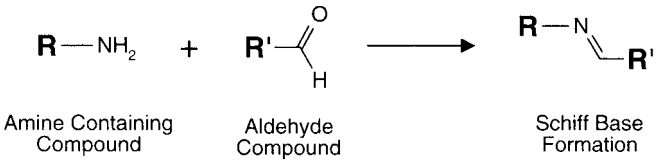
Derivatives of hydrazine, especially the hydrazide compounds formed from carboxylate groups, can react specifically with aldehyde or ketone functional groups in target molecules. Reaction with either group creates a hydrazone linkage (Reaction 44)—a type of Schiff base. This bond is relatively stable if it is formed with a ketone, but somewhat labile if the reaction is with an aldehyde group. However, the reaction rate of hydrazine derivatives with aldehydes typically is faster than the rate with ketones. Hydrazone formation with aldehydes, however, results in much more stable bonds than the easily reversible Schiff base interaction of an amine with an aldehyde. To further stabilize the bond between a hydrazide and an aldehyde, the hydrazone may be reacted with sodium cyanoborohydride to reduce the double bond and form a secure covalent linkage.



(Reaction 44)

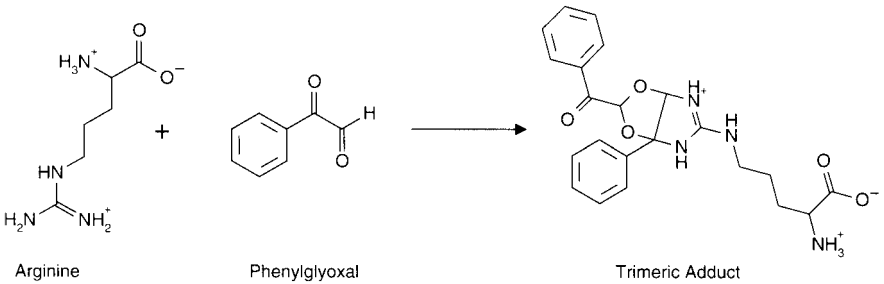
5.2. Schiff Base Formation

Aldehydes and ketones can react with primary and secondary amines to form Schiff bases, a dehydration reaction yielding an imine (Reaction 45). However, Schiff base formation is a relatively labile, reversible interaction that is readily cleaved in aqueous solution by hydrolysis. The formation of Schiff bases is enhanced at alkaline pH values, but they are still not stable enough to use for crosslinking applications unless they are reduced by reductive amination (see below).



(Reaction 45)

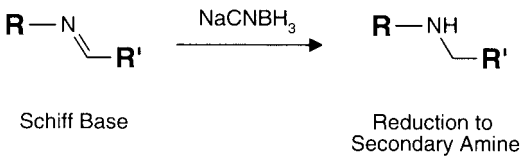
The reaction of dicarbonyl compounds, such as glyoxal or phenylglyoxal, with a guanidinyl group, such as that of an arginine residue, proceeds to yield a more stable linkage due to the formation of a cyclic derivative (Reaction 46).



(Reaction 46)

5.3. Reductive Amination

Reductive amination (or alkylation) may be used to conjugate an aldehyde or ketone containing molecule with an amine-containing molecule. Schiff base formation between aldehydes and amines occurs readily in aqueous solutions, especially at elevated pH. This type of linkage, however, is not stable unless reduced to secondary or tertiary amine bonds. A number of reducing agents can be used to convert specifically the Schiff base interaction into an alkylamine linkage (Reaction 47). Once reduced, the bonds are highly stable and will not readily hydrolyze in aqueous environments. The use of reductive amination to conjugate an aldehyde containing molecule to an amine containing molecule results in a zero-length crosslinking procedure where no additional spacer atoms are introduced between the molecules (Chapter 3, Section 4). Reaction of ammonia or a diamine compound with an aldehyde by reductive amination is a method of creating a primary amine functional group (Chapter 1, Section 4.3).

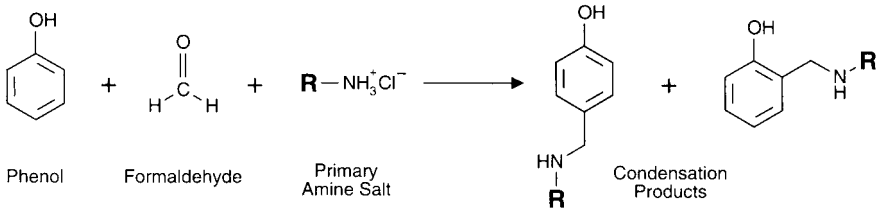


(Reaction 47)

5.4. Mannich Condensation

Aldehydes may participate in a condensation reaction with an amine compound and a substance containing a sufficiently-active hydrogen, yielding an alkylated derivative that effectively

crosslinks the two molecules through the carbonyl group of the aldehyde. Strictly speaking, the Mannich reaction consists of the condensation of formaldehyde (or sometimes another aldehyde) with ammonia, in the form of its salt, and another compound containing an active hydrogen. Instead of using ammonia, however, this reaction can be done with primary or secondary amines, or even with amides. An example is illustrated in the condensation of phenol, formaldehyde, and a primary amine salt (Reaction 48).



(Reaction 48)

The Mannich reaction provides an often-superior alternative to diazonium conjugation (Section 6.1, this chapter), because of the disadvantages inherent in the instability of both the diazonium group and the resultant diazo linkage. By contrast, conjugations done through Mannich condensations result in stable covalent bonds.

The crosslinking scheme using this method can make use of the native ε- and N-terminal amines on proteins as the source of primary amine for the condensation reaction. Added to the conjugation reaction is formaldehyde and the desired molecule to be coupled containing an appropriately active hydrogen. The Mannich reaction should not be used for molecules containing both an amine and a reactive hydrogen, since polymerization may occur. It is especially useful for preparing hapten–carrier conjugates when the hapten contains no other available functionalities suitable for crosslinking, but does contain an active hydrogen (Chapter 19, Section 6.2).

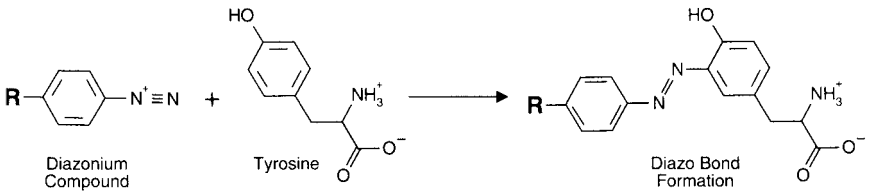
6. Active Hydrogen Reactions

Many compounds contain reactive (or replaceable) hydrogens that are able to participate in conjugation procedures using certain chemical reactions. These hydrogens typically are associated with aromatic systems wherein an electron donating group activates positions on the ring toward substitution reactions. At such carbons, the hydrogen is easily displaced by an attacking electrophilic group able to form a new covalent linkage. Several common modification reactions are used in bioconjugate chemistry to label or crosslink molecules at active hydrogen sites. The following three sections discuss these chemical reactions.

6.1. Diazonium Derivatives

Diazonium groups react with active hydrogen sites on aromatic rings to give covalent diazo bonds. Generation of a diazonium functional group usually is done from an aromatic amine

by reaction with sodium nitrite under acidic conditions at 0°C (Chapter 1, Section 4.3 and Chapter 19, Section 6.1). The highly reactive and unstable diazonium is reacted immediately with an active hydrogen-containing compound at pH 8–10. In general, at pH 8.0 the diazonium group will react principally with histidinyl residues, attacking the electron-rich nitrogens of the imidazole ring. At higher pH, the phenolic side chain of tyrosine groups can be modified (Reaction 49). The reaction proceeds by electrophilic attack of the diazonium group toward the electron rich points on the target molecules. Phenolic compounds are modified at positions *ortho* and *para* to the aromatic hydroxyl group. For tyrosine side chains, only the *ortho* modification is available.



(Reaction 49)

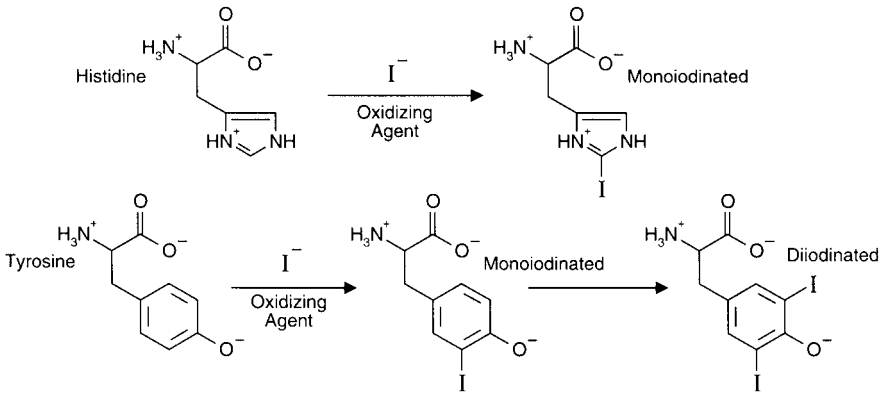
Crosslinking using diazonium compounds usually creates deeply colored products characteristic of the diazo bonds. Occasionally, the conjugated molecules may turn dark brown or even black. The diazo linkages are reversible by addition of 0.1 M sodium dithionite in 0.2 M sodium borate, pH 9.0. Upon cleavage, the color of the complex is lost.

6.2. Mannich Condensation

The Mannich reaction consists of the condensation of an active hydrogen-containing compound with an amine-containing compound in the presence of formaldehyde. See Section 5.4 (this chapter) for addition details.

6.3. Iodination Reactions

Radioiodination involves the substitution of radioactive iodine atoms for reactive hydrogen sites in target molecules. The process usually involves the action of a strong oxidizing agent to transform iodide ions into a highly reactive electrophilic iodine compound (typically I_2 or a mixed halogen species such as ICl). Formation of this electrophilic species leads to the potential for rapid iodination of aromatic compounds containing strong activating groups, such as aryl compounds. Particularly, aromatic constituents that have electron donating groups can sufficiently activate the carbons on the ring to undergo electrophilic substitution reactions. Therefore, phenols, aniline derivatives, or alkyl anilines that contain OH, NH_2, or NHR constituents, respectively, are very susceptible to being iodinated. In proteins, this translates into tyrosine side chain phenolic groups and histidine side chain imidazole groups (Reaction 50). See Chapter 12 for further details on iodination reactions.



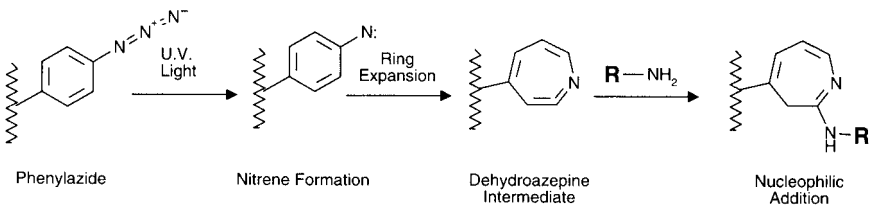
(Reaction 50)

7. Photo-Chemical Reactions

Photoreactive groups can be induced to couple with target molecules by exposure to UV light. Until they are photolyzed, photosensitive functional groups are relatively non-reactive in typical thermochemical processes. For this reason, reagents designed with a photoreactive group can be used in highly controlled reactions. The labeling reaction can be induced by a UV flash at predetermined points in an experimental protocol. For instance, covalent bond formation can be initiated after binding of photo-labeled ligands to receptors or after some other biochemical process has taken place. In this regard, photoreactive chemistry has become an important device for numerous bioconjugate applications. The following sections describe the major photosensitive groups that can be used in the design of modification or crosslinking reagents. (Chapter 4, Section 5; Chapter 5, Sections 3–7; Chapter 6; and Chapter 11, Section 4 describe the reagents that utilize these functional groups.)

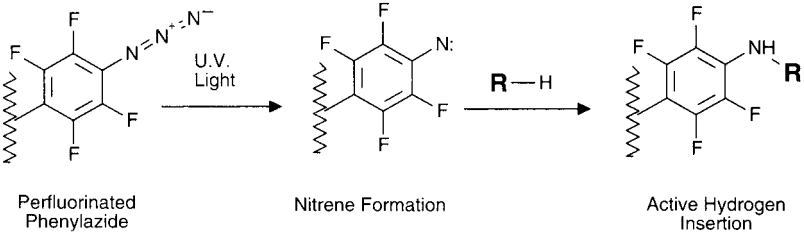
7.1. Aryl Azides and Halogenated Aryl Azides

The most popular type photosensitive functional group is the aryl azide derivative. Upon photolysis, phenyl azide groups form short-lived nitrenes that react rapidly with the surrounding chemical environment (Gilchrist and Rees, 1969). Nitrenes can insert nonspecifically into chemical bonds of target molecules, including undergoing addition reactions with double bonds and insertion reactions into active hydrogen bonds at C—H and N—H sites. Abundant evidence, however, indicates that the photolyzed intermediates of aryl azides principally undergo ring expansion to create nucleophile-reactive dehydroazepines. Instead of inserting non-selectively at active carbon–hydrogen bonds, dehydroazepines have a tendency to react preferentially with nucleophiles, especially amines (Reaction 51).



(Reaction 51)

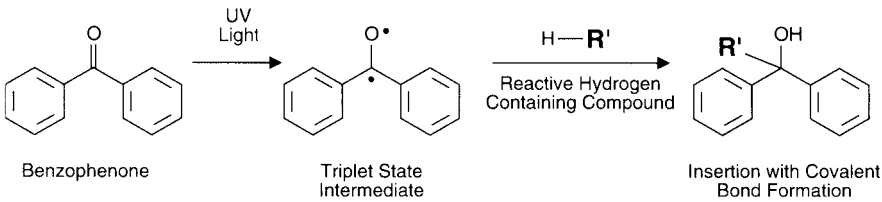
However, some investigators have shown that aryl azides that possess a perfluorinated ring structure or are substituted completely with halogen atoms are quite efficient at forming the desired nitrene intermediate (Keana and Cai, 1990; Cai *et al.*, 1993; Schnapp and Platz, 1993; Soundararajan *et al.*, 1993; Schnapp *et al.*, 1993; Yan *et al.*, 1994). The ring substitution prevents ring expansion after nitrene formation, thus allowing the reactive intermediate to survive long enough to react with target molecules. Halogenated phenyl azides undergo the insertion reactions that were typically attributed to unsubstituted aryl azides in the past (Reaction 52).



(Reaction 52)

7.2. Benzophenones

A photoreactive group consisting of a benzophenone residue photolyzes upon exposure to UV light to give a highly reactive triplet-state ketone intermediate (Walling and Gibian, 1965). Similar to the reactive nitrene of photolyzed phenyl azides, the energized electron of an activated benzophenone can insert in hydrogen–carbon bonds and other active groups to give covalent linkages with target molecules (Reaction 53). Unlike phenyl azides, however, the decomposition or decay of the photoactivated species does not yield an inactive compound. Instead, benzophenones that have become deactivated without forming a covalent bond can be once again photolyzed to an active state. As a result of this multiple-activation characteristic, a benzophenone reagent has more than one chance to form a covalent bond with its intended target. Thus it typically gives much higher yields of photo-crosslinking than comparable phenyl azide crosslinkers.



(Reaction 53)

The use of a benzophenone photoactivatable group in the design of bioconjugate reagents is rare. Two sulfhydryl-reactive ones incorporating a maleimide group and a iodoacetyl group opposite the benzophenone are described in Chapter 5, Sections 4.3 and 4.4. A newer benzophenone crosslinker containing a water-soluble PEG spacer is described in Chapter 18, Section 3.6.

7.3. Anthraquinones

Anthraquinone groups are highly photoreactive by exposure to long UV light ranging from 340 to 360 nm. Unlike photoreactive groups that form intermediate nitrene or carbine precursors

after photoactivation, anthraquinones react by a radical generation process, which is much more efficient in coupling to C—H substrates. Photoactivation results in a highly reactive, excited species that involves the formation of a triplet n,π^*-state, which becomes a powerful electron acceptor. If an organic substrate is present containing a reactive C—H bond, then the excited anthraquinone is able to cause rapid proton abstraction, resulting in the formation of a reduced, phenoxy radical intermediate with a second radical formed on the hydrogen donating substrate. This radical pair then can react to link covalently the anthraquinone group to the substrate, forming an ether linkage and effectively immobilizing the reagent to the substrate (Brennan and Beutel, 1969; Koch *et al.*, 2000) (Figure 2.6).

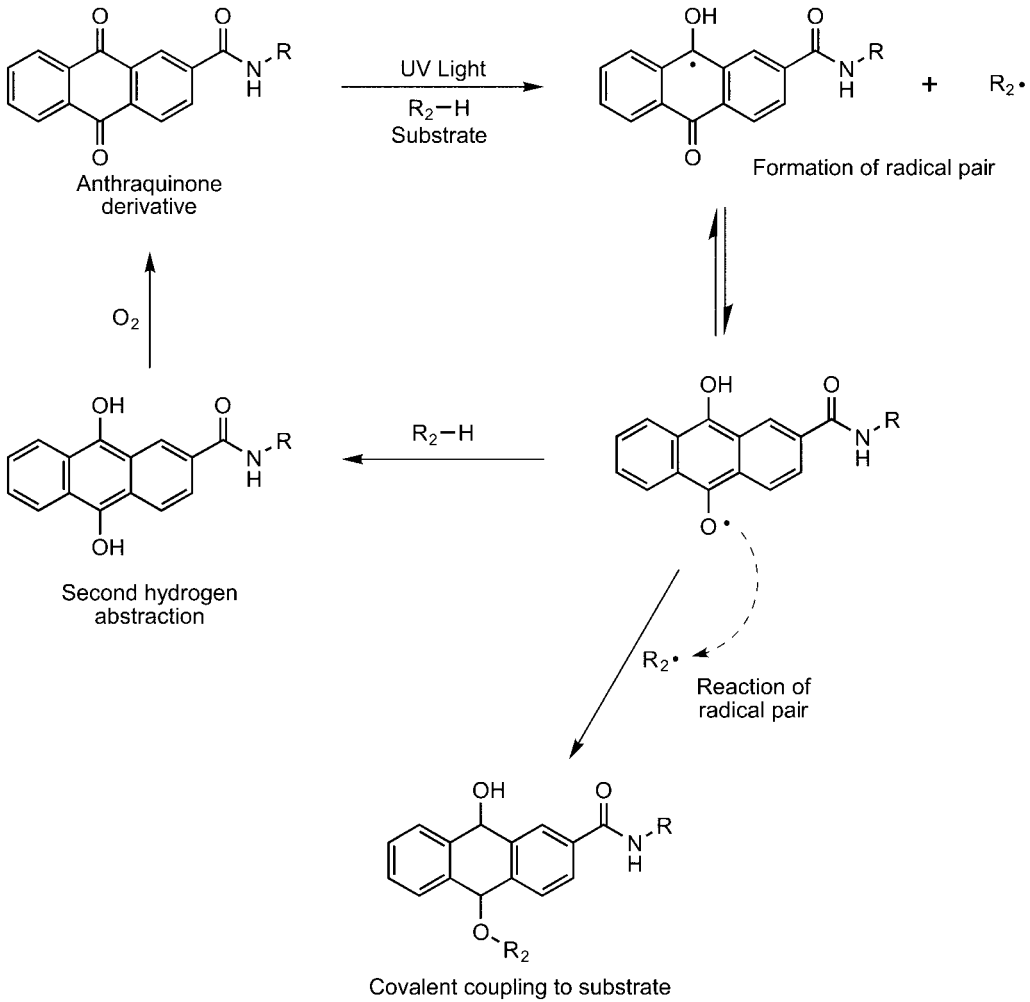
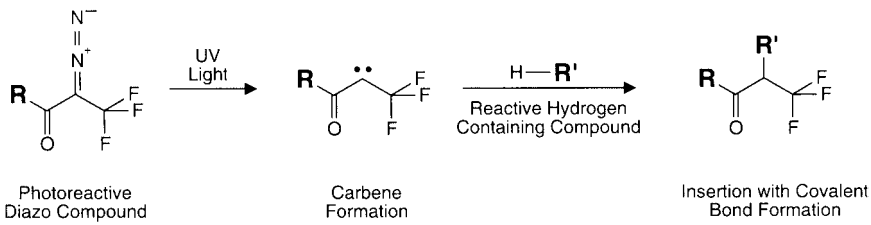


Figure 2.6 Anthraquinone derivatives can photoreactively couple to substrates by means of a free radical generation process. The reactive intermediate also can be regenerated back to the initial anthraquinone by proton abstraction and oxidation, resulting in the possibility of again being photolyzed and successfully coupled to the substrate.

Modification or crosslinking reagents containing an anthraquinone group can be made from the 2-carboxylic acid derivative (Kumar *et al.*, 2004) (Exiqon). A spacer arm can be added to the carboxylate to terminate in a reactive group for bioconjugation or an affinity group (e.g., biotin) for interaction with other molecules. Anthraquinone photoreactive linking reagents can be used to modify any surfaces or particles containing C—H groups, including polymer-based microplates, slides, and particles. Inorganic surfaces can be modified with an organosilane compound (Chapter 13) and then further reacted using an anthraquinone compound, however the benefit of this strategy may be negated by choice of the proper reactive group on the silane. Reactions with surfaces usually are done with anthraquinone concentrations in aqueous buffers ranging from 100 ng/ml to about 2 μg/ml. Protect all photoreactive reagents and solutions from light until ready to photoactivate them.

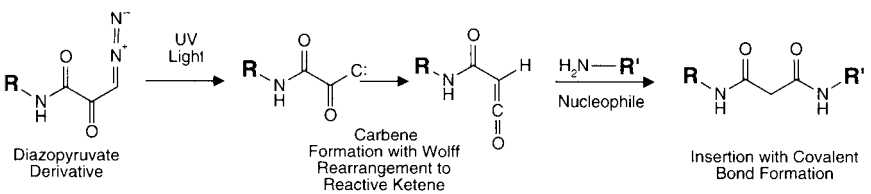
7.4. Certain Diazo Compounds

Certain diazo compounds can be photolyzed with UV light to generate highly reactive carbenes (Reaction 54). Similar to nitrenes, carbenes can insert into active C—H or N—H bonds or add to double bonds, forming covalent linkages with target molecules (Gilchrist and Rees, 1969). Few diazo photoreactive reagents have been synthesized, probably due to their tendency to react with water molecules after photoactivation, thus severely decreasing coupling yields with intended molecules. One heterobifunctional crosslinker, PNP-DTP, containing an amine-reactive end and a photosensitive diazotrifluoropropionate group is available (Chapter 5, Section 3.12).



(Reaction 54)

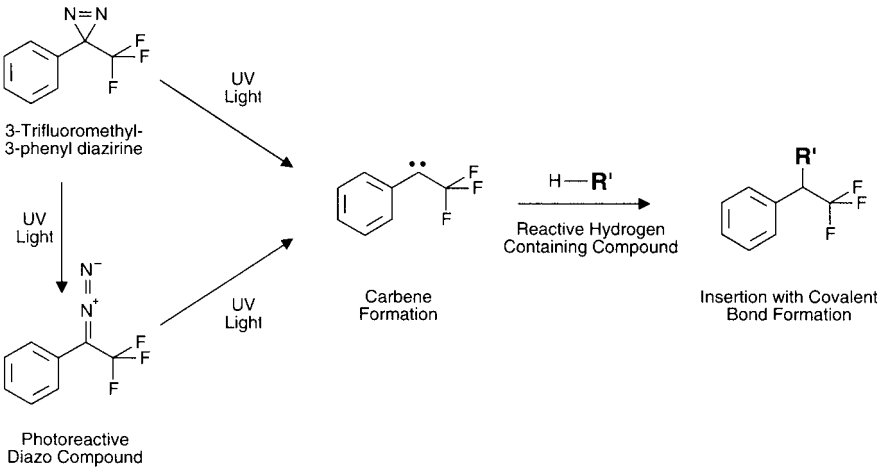
Diazopyruvates are another class of photoreactive diazo compounds that have a unique coupling mechanism (Chapter 5, Section 3.11). The diazo functional group can by photolyzed by exposure to irradiation at 300 nm, forming a highly reactive carbene, which can undergo a Wolff rearrangement to produce a ketene amide intermediate. In the presence a nucleophilic species on a target molecule, the ketene can undergo an acylation reaction to form a stable malonic acid derivative. The photolyzed product thus can couple to hydrazide or amine containing targets to form covalent linkages (Reaction 55).



(Reaction 55)

7.5. Diazirine Derivatives

Diazirine compounds are similar in their photoreactivity to diazo groups, forming highly reactive carbene intermediates upon exposure to UV light of about 360 nm (Reaction 56). Diazirines consist of a three-member ring system containing two nitrogen atoms connected through a double bond. First developed by Smith and Knowles (1973), the photosensitive diazirine is perhaps second in popularity to phenyl azides in the design of photoreactive crosslinking agents.



(Reaction 56)

Some diazirines, particularly the 3-trifluoromethyl-3-aryldiazirines, can rearrange upon photolysis to a linear diazo derivative, similar in structure to the photosensitive end of the crosslinker PNP-DTP (Chapter 5, Section 3.12). These isomerized products themselves can be photolyzed to the reactive carbene.

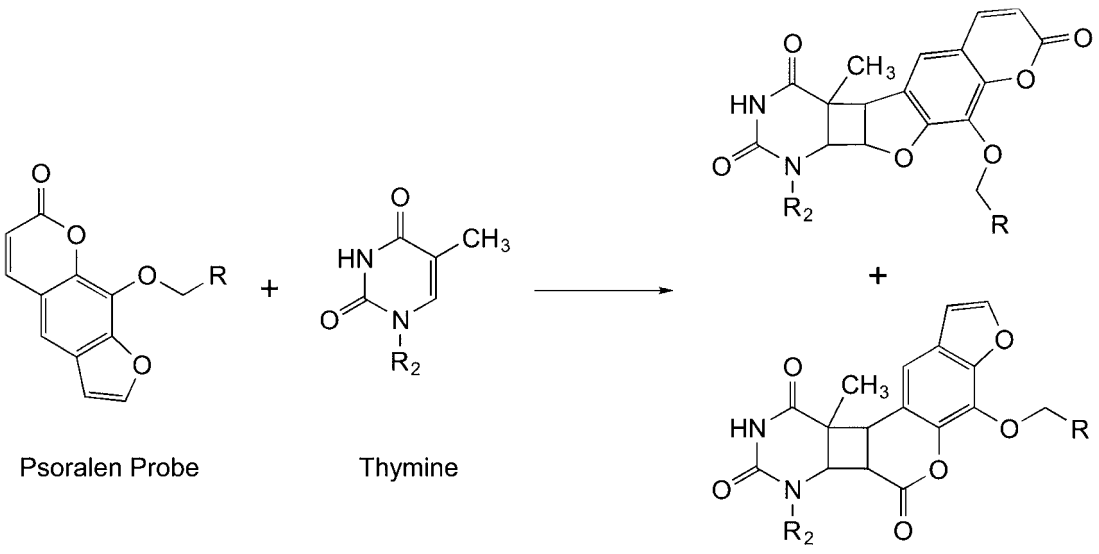
Carbene generation from photolysis of diazirine compounds leads to efficient insertion into C—H or N—H bonds and also causes addition reactions with points of unsaturation within target molecules. Diazirine-containing photoaffinity probes have been used to study numerous ligand–receptor interactions (Bergmann *et al.*, 1994). Heterobifunctional crosslinkers containing a diazirine photosensitive group also have been used to attach macromolecules to surfaces such as polystyrene and glass (Collioud *et al.*, 1993). In addition, diazirine-containing photoreactive amino acid analogs, photo-leucine and photo-methionine, have been developed to study protein interactions within cells (Suchanek *et al.*, 2005).

7.6. Psoralen Compounds

Psoralen, or derivatives of 9-methoxy-7*H*-furo[3,2-*g*]chromen-7-one tricyclic ring structures, are used as photoreactive groups in crosslinkers, biotinylation compounds, and nucleic acid probes. Psoralens have been used for many years as photochemotherapy agents for treatment of psoriasis and vitiligo (Smith and Barker, 2006). Psoralens react when exposed to UV light

ranging from 320 to 400 nm to form an excited triplet state intermediate that can insert in certain double bond structures, especially at the 5,6-double bond of thymine bases.

Psoralens can react by two different routes upon photoactivation (Parsons, 1980; Pathak, 1984). The first route is through the well-known photoreaction mechanism that principally involves intercalation within double-stranded DNA or RNA with the formation of adducts with adjacent thymine bases. The furan-side and pyrone-side rings in psoralen both can form cycloaddition products with the 5,6-double bond of thymine to create a crosslink between two DNA strands (Reaction 57) or to a lesser extent, within double-strand regions of RNA.



(Reaction 57)

Psoralen also can undergo reactions with oxygen to produce reactive oxygen species, including the formation of singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radicals ($^{\bullet}OH$). The production of reactive oxygen species by psoralen derivatives can damage biological molecules and structures. See Chapter 1, Section 1.1 for additional information on the oxidation of amino acids.

Psoralen-biotin compounds have been used to label double-stranded DNA for detection using (strept)avidin reagents (Henriksen *et al.*, 1991; Wygrecka *et al.*, 2007). The compound psoralen-PEG_3-biotin is commercially available for this purpose (Thermo Fisher). Crosslinking agents also can be built using a photoreactive psoralen ring system at one end. The reagent succinimidyl-[4-(psoralen-8-yloxy)]butyrate (SPB) contains an NHS ester to covalently link a psoralen group to proteins or other amine-containing molecules (Thermo Fisher, Molecular Biosciences). Oser *et al.* (1988) created a lanthanide chelate with a psoralen group to label DNA with a time-resolved fluorescent probe. Psoralen derivatives also can be coupled to polymeric surfaces by a

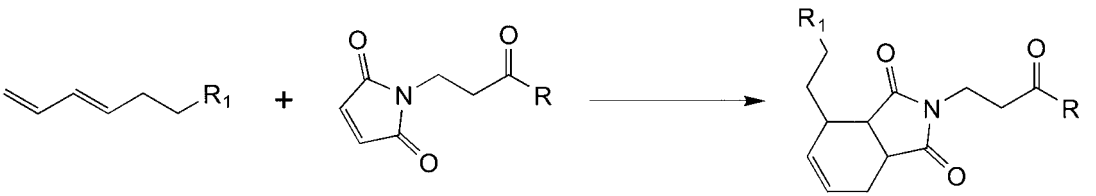
photoreaction process. Elsner and Mouritsen (1994) used psoralen linkers for modification of the surface of microplates to create sites for covalent binding of affinity molecules.

8. Cycloaddition Reactions

The following sections briefly describe three cycloaddition reactions that can be used to form bioconjugates. These reactions represent highly specific reactant pairs that have a chemoselective nature, meaning they mainly react with each other and not other functional groups, such as those found on biomolecules. For a complete discussion of chemoselective ligation reactions, see Chapter 17.

8.1. Diels–Alder Reaction

The Diels–Alder reaction consists of the covalent coupling of a diene with an alkene to form a 6-membered ring complex. This process has been used extensively in organic synthesis, but only recently has it been applied to bioconjugation reactions (Hill *et al.*, 2001). This reaction proceeds at room temperature or slightly elevated temperature conditions (30°C) to give the 2 + 4 cycloaddition product, a hexane ring containing a single double bond. The reaction can be done using a maleimide group as the alkene derivative and a hexadienyl group as the diene (Reaction 58). The reaction process can give 90–95 percent yields in 1–18 hours.



(Reaction 58)

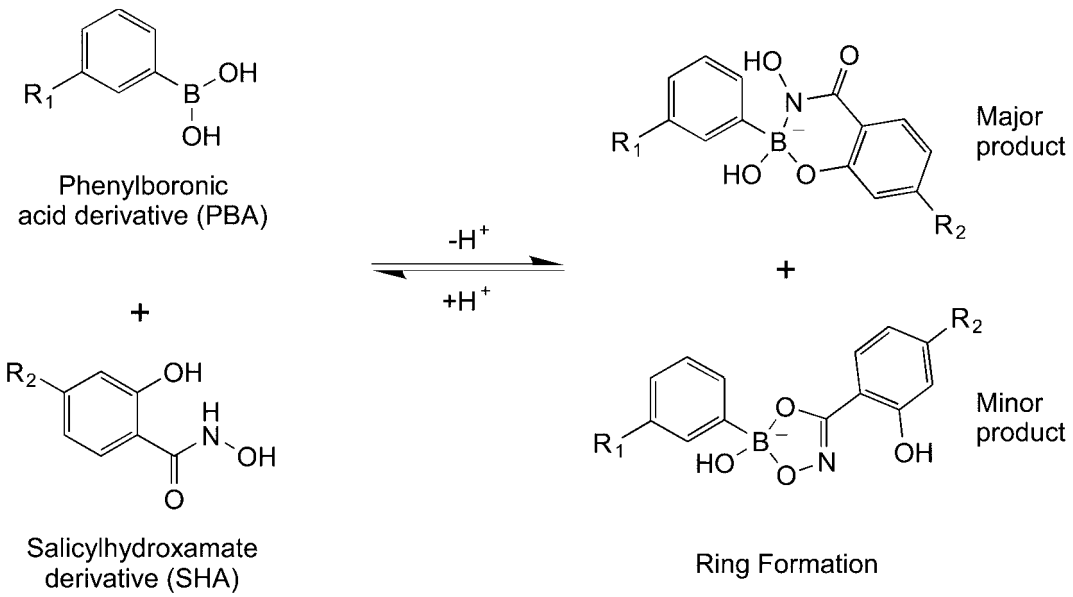
See Chapter 17, Section 1 for additional information concerning the use of the Diels–Alder reaction in bioconjugation applications.

8.2. Complex Formation with Boronic Acid Derivatives

Boronic acid derivatives are able to form ring structures with other molecules having neighboring functional groups consisting of 1,2- or 1,3-diols, 1,2- or 1,3-hydroxy acids, 1,2- or 1,3-hydroxylamines, 1-2- or 1,3-hydroxyamide, 1,2- or 1,3-hydroxyoxime, as well as various

sugars containing these species (Weith *et al.*, 1970; Rosenberg and Gilham, 1971; Rosenberg *et al.*, 1972; Pace and Pace, 1980; Singhal *et al.*, 1980). The products of these reactions are 5- or 6-membered heterocyclic rings, which in some cases are reversible with a change in pH or by the addition of a counter-ligand having competing functional groups.

Typically, the boronic acid group is part of an aminophenyl boronic acid derivative, and this group has been used for bioconjugation and affinity chromatography purposes (Burnett *et al.*, 1980; O'Shannessy and Quarles, 1987). A common partner for a phenyl boronic acid group in bioconjugation is the salicylhydroxamic acid (SHA) group (Chapter 17, Section 3) (Reaction 59).

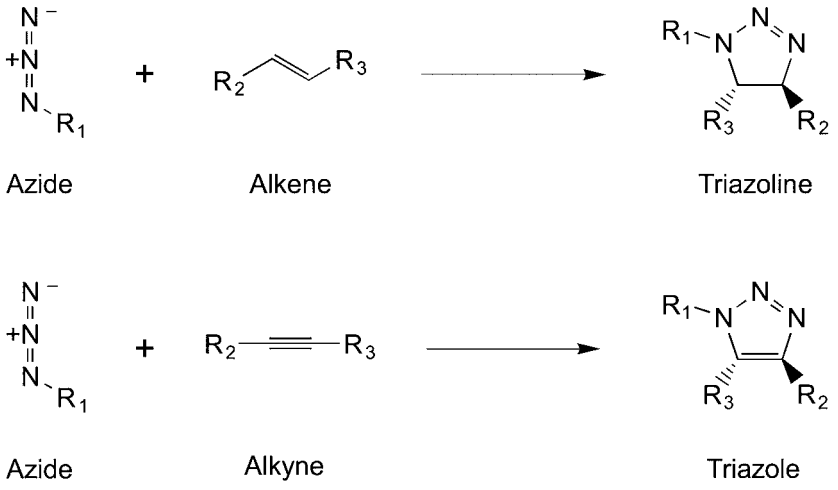


(Reaction 59)

8.3. Click Chemistry: Cu^{1}-promoted Azide—Alkyne [3 + 2] Cycloaddition

Click chemistry refers to the reaction between an azido functional group and an alkyne to form a [3 + 2] cycloaddition product, a 5-membered triazole ring. This reaction has been used for many years in organic synthesis to form heterocyclic rings. Normally, the click reaction requires high temperatures, and this was the main reason that it was not used as a bioconjugation tool. However, it was discovered that in aqueous solutions and in the presence of Cu(I), the reaction kinetics are dramatically accelerated to provide high yields even at room temperature and ambient pressures (Rostovtsev *et al.*, 2002; Tornøe *et al.*, 2002; Sharpless *et al.*, 2005).

The advantage of the click reaction for bioconjugation is that the reactant pair is not reactive with any other functional group encountered in biological systems. This property of



(Reaction 60)

bioorthogonality provides extreme selectivity for bringing together azide and alkyne derivatives to form triazoles even in complex biological samples.

Reaction 60 shows the reaction of an alkyne with an azide to form a triazole ring in the presence of a Cu(I) catalyst. See Chapter 17, Section 4 for additional details on the use of this conjugation reaction.

PART II

Bioconjugate Reagents

The reagent systems used in bioconjugate procedures are as varied as their intended applications. Whether it is for tagging proteins to make them chromogenic or fluorescent, labeling molecules with biospecific ligands for subsequent affinity interactions, or crosslinking two or more substances to create uniquely active conjugates, the choice of reagents available for use is limited only by the imagination.

Over the last 30 years, the selection of crosslinking and modifying agents has grown not only in shear number, but in the availability of novel reactive groups and in the variety of their design. Today, regardless of the particular need, a workable reagent system that will yield a useful derivative almost always can be found. The best and most effective of the reported reagent systems usually are available from commercial sources, and thus do not even have to be synthesized.

In Part II, the reagents of modification and conjugation have been categorized according to structural type, reactivity, and use. Where possible and appropriate, generalized protocols have been provided for each reagent's most likely application. The options described herein, combined with a thorough knowledge of the basic chemical reactions that their functional groups provide (as discussed in Part I), allow the creation of an intelligent design and plan of attack for any desired application. The labeling, tagging, crosslinking, or targeting of small ligands, peptides, proteins, carbohydrates, nucleic acids, oligonucleotides,

lipids, and a host of other compounds may be accomplished by the judicious choice of the appropriate reagent system.

The following reagents have been used in everything from bench-scale experiments in research laboratories to process-optimized applications in the diagnostic and therapeutic industries. Conjugated or modified molecules have been applied in procedures designed to visualize target substances, as key components in clinical assay systems, and in the latest affinity-directed therapeutics, such as anti-tumor immunotoxins. Some of the reagent systems described in Part II have formed the basis for literally a multi-billion dollar biotechnology industry.

3

Zero-Length Crosslinkers

The smallest available reagent systems for bioconjugation are the so-called zero-length crosslinkers. These compounds mediate the conjugation of two molecules by forming a bond containing no additional atoms. Thus, one atom of a molecule is covalently attached to an atom of a second molecule with no intervening linker or spacer. In many conjugation schemes, the final complex is bound together by virtue of chemical components that add foreign structures to the substances being crosslinked. In some applications, the presence of these intervening linkers may be detrimental to the intended use. For instance, in the preparation of hapten–carrier conjugates the complex is formed with the intention of generating an immune response to the attached hapten. Occasionally, a portion of the antibodies produced by this response will have specificity for the crosslinking agent used in the conjugation procedure. Zero-length crosslinking agents eliminate the potential for this type of cross-reactivity by mediating a direct linkage between two substances.

The reagents described in this section can initiate the formation of three types of bonds: an amide linkage made by the condensation of a primary amine with a carboxylic acid, a phosphoramidate linkage made by the reaction of an organic phosphate group with a primary amine, and a secondary or tertiary amine linkage made by the reductive amination of a primary or secondary amine with an aldehyde group. Therefore, using these reagent systems, substances containing amines can be conjugated with other molecules containing phosphates or carboxylates. Alternatively, substances containing amines can be crosslinked to molecules containing formyl groups. All of the reactions are quite efficient, and depending on the reagent chosen and the desired application, they may be performed in aqueous or nonaqueous environments.

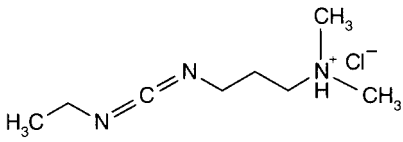
1. Carbodiimides

Carbodiimides are used to mediate the formation of amide linkages between carboxylates and amines or phosphoramidate linkages between phosphates and amines (Hoare and Koshland, 1966; Chu *et al.*, 1986; Ghosh *et al.*, 1990). They are probably the most popular type of zero-length crosslinker in use, being efficient in forming conjugates between two protein molecules, between a peptide and a protein, between an oligonucleotide and a protein, between a biomolecule and a surface or particle, or any combination of these with small molecules. There are two basic types of carbodiimides: water-soluble and water-insoluble. The water-soluble ones are

the most common choice for biochemical conjugations, because most macromolecules of biological origin are soluble in aqueous buffer solutions. Not only is the carbodiimide itself able to dissolve in the reaction medium, but the by-product of the reaction, an isourea, is also water-soluble, facilitating easy purification. Water-insoluble carbodiimides, by contrast, are used frequently in peptide synthesis and other conjugations involving molecules soluble only in organic solvents. Both the organic-soluble carbodiimides and their isourea by-products are insoluble in water.

1.1. EDC

EDC (or EDAC; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) is the most popular carbodiimide used for conjugating biological substances containing carboxylates and amines. In fact, it also may be the most frequently used crosslinking agent of all. Its application in particle and surface conjugation procedures along with NHS (*N*-hydroxysulfosuccinimide) or sulfo-NHS is nearly universal (Chapter 14) and this fact makes it the most common bioconjugation reagent in use today. EDC is water-soluble, which allows for its direct addition to a reaction without prior organic solvent dissolution. Both the reagent itself and the isourea formed as the by-product of the crosslinking reaction are water-soluble and may be removed easily by dialysis or gel filtration (Sheehan *et al.*, 1961, 1965). The reagent is, however, labile in the presence of water. The bulk chemical should be stored desiccated at −20°C. Warm the bottle to room temperature before opening to prevent condensation occurring that will cause decomposition of the reagent over time. A concentrated solution of EDC in water may be prepared to facilitate the addition of a small molar amount to a reaction, but the stock solution should be dissolved rapidly and used immediately to prevent extensive loss of activity.



EDC
1-Ethyl-3-(3-dimethylaminopropyl)
Carbodiimide Hydrochloride
MW 191.7

A variety of chemical conjugates may be formed using EDC (Chu *et al.*, 1976, 1982; Chu and Ueno, 1977; Yamada *et al.*, 1981; Chase *et al.*, 1983), provided one of the molecules contains an amine and the other a carboxylate group. *N*-substituted carbodiimides can react with carboxylic acids to form highly reactive, *o*-acylisourea intermediates (Figure 3.1). This active species then can react with a nucleophile such as a primary amine to form an amide bond (Williams and Ibrahim, 1981). Other nucleophiles are also reactive. Sulfhydryl groups may attack the active species and form thiol ester linkages, although these are not as stable as the bond formed with an amine. In addition, oxygen atoms may act as the attacking nucleophile, such as those in water molecules. In aqueous solutions, hydrolysis by water is the major competing reaction, cleaving off the activated ester intermediate, forming an isourea, and regenerating the carboxylate group (Gilles *et al.*, 1990).

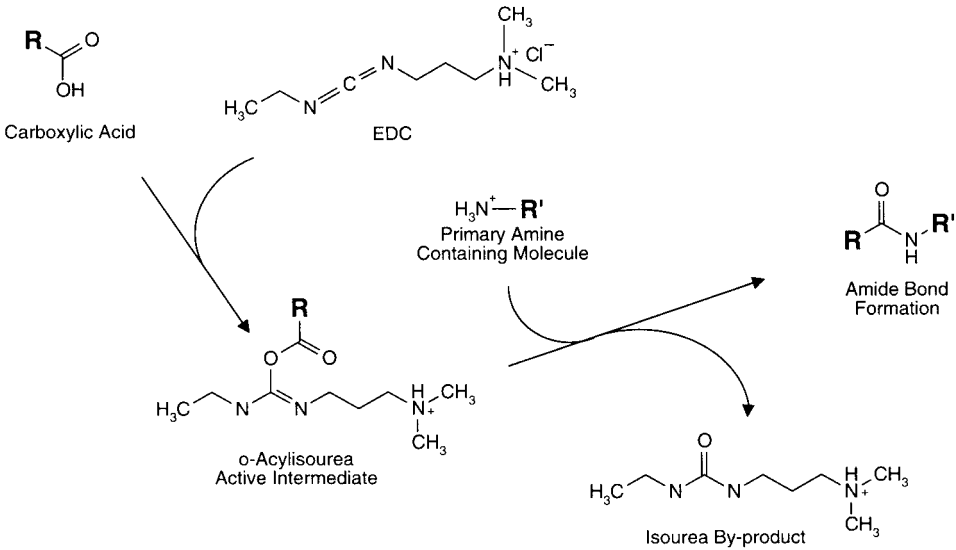


Figure 3.1 EDC reacts with carboxylic acids to create an active-ester intermediate. In the presence of an amine nucleophile, an amide bond is formed with release of an isourea by-product.

Nakajima and Ikada (1995) investigated the reactions of EDC amide bond formation in aqueous solution using hydrogels of acrylic acid- or maleic acid-containing polymers or other carboxylate molecules to contribute the activatable groups and ethylene diamine or benzylamine as the amine functional groups to be conjugated. Their results indicate that carboxylate activation occurs most effectively with EDC at pH 3.5–4.5, while amide bond formation occurs with highest yield in the range of pH 4–6. However, EDC hydrolysis occurs maximally at acidic pH values with increasing stability of the carbodiimide in solution at or above pH 6.5. When working with proteins and peptides, experience indicates that EDC-mediated amide bond formation effectively occurs between pH 4.5 and 7.5. Beyond this pH range, however, the coupling reaction occurs more slowly with lower yields.

The presence of both carboxylates and amines on one of the molecules to be conjugated with EDC may result in self-polymerization, because the substance then can react with another molecule of its own kind instead of the desired target. For instance, when conjugating peptides to carrier proteins using EDC, the peptide usually contains both a carboxylate and an amine. The result typically is peptide polymerization in addition to coupling to the carrier (see Chapter 19, Section 3). For this type of immunogen conjugation, polymerization is not usually detrimental to its use, because polymerized peptide is also immunogenic. However, for other crosslinking applications where it may be more desirable to avoid oligomer formation, the use of a carbodiimide may not be the best choice of reagent, especially if one of the molecules being conjugated contains both a carboxylate and an amine.

Most references to the use of EDC describe the optimal reaction medium to be at a pH from 4.7 to 6.0. However, the carbodiimide reaction occurs effectively up to at least pH 7.5 without significant loss of yield. Conjugations done under mildly alkaline pH conditions (e.g., pH 8.5) also can be done to limit the polymerization of proteins, while still facilitating the coupling of a carboxylate-containing molecule at a low substitution level per protein. See Chapter 19,

Section 3 for additional information on the properties of EDC conjugation using small peptides coupled to carrier proteins.

Some procedures recommend the use of water as the solvent in an EDC reaction, while the pH is maintained constant by the addition of HCl. Buffered solutions are more convenient, because the pH does not have to be monitored during the course of the reaction. For acidic pH conjugations, MES [2-(*N*-morpholino)ethane sulfonic acid] buffer at 0.1 M works well. When doing neutral pH reactions, a phosphate buffer at 0.1 M is appropriate. Any buffers may be used that do not interfere with the reaction, but avoid amine- or carboxylate-containing buffer salts or other components in the medium that may react with the carbodiimide.

There are some side reactions that may occur when using EDC with proteins. In addition to reacting with carboxylates, EDC itself can form a stable complex with exposed sulfhydryl groups (Carraway and Triplett, 1970). Tyrosine residues can react with EDC, most likely through the phenolate ionized form of its side chain (Carraway and Koshland, 1968). The imidazolyl group of histidine may react with sulfo-NHS esters, resulting in an active carbonyl imidazole group which subsequently hydrolyzes (Cuatrecasas and Parikh, 1972). Finally, EDC may promote unwanted polymerization due to the usual abundance of both amines and carboxylates on protein molecules.

The following protocol is a generalized description of how to conjugate a small amine- or carboxylate-containing molecule to a protein. The protocol may be modified by changing the pH, buffer salts, and ratios of reactants to obtain the desired product. Specific protocols utilizing EDC in selected conjugation applications may be found in Part III. In some cases, the parameters of this generalized protocol may have to be modified to retain solubility or activity of the resulting conjugate. For instance, coupling hydrophobic molecules to the surface of proteins often causes partial or complete precipitation. This problem may be somewhat alleviated by decreasing either the amount of EDC or the amount of the hydrophobic molecule added to the reaction, thus resulting in a lower density of substitution. Protocols on the use of EDC to couple proteins or other molecules to particles may be found in Chapter 14 and Chapter 9, Section 10.

Protocol

1. Dissolve the protein to be modified at a concentration of 10 mg/ml in one of the following reaction media: (a) water, (b) 0.1 M MES, pH 4.7–6.0, or (c) 0.1 M sodium phosphate, pH 7.3. NaCl may be added (i.e., 0.15 M) if desired. If lower or higher concentrations of the protein are used, adjust the amounts of the other reactants added as necessary to maintain the correct molar ratios. For the preparation of a peptide–protein immunogen conjugate, a 200 µl solution of the carrier protein at a concentration of 10 mg/ml in 0.1 M MES, pH 4.7 usually works well.
2. Dissolve the molecule to be coupled in the same buffer used in step 1. For small molecules, add them to the reaction in at least a 10-fold molar excess to the amount of protein present. If possible, the molecule may be added directly to the protein solution in the appropriate excess. Alternatively, dissolve the molecule in the buffer at a higher concentration, and then add an aliquot of this stock solution to the protein solution. In the example of preparing a peptide–protein conjugate, dissolve the peptide in 0.1 M MES, pH 4.7, at a concentration of up to 2 mg/500 µl.
3. Add the solution prepared in step 2 to the protein solution to obtain at least a 10-fold molar excess of small molecule to protein. In the case of the peptide–protein immunogen conjugate, add the 500 µl of peptide solution to the 200 µl of protein solution.

4. Add EDC (Thermo Fisher) to the above solution to obtain at least a 10-fold molar excess of EDC to the protein. Alternatively, a 0.5–0.1 M EDC concentration in the reaction mixture usually works well. To make it easier to add the correct quantity of EDC, a higher concentration stock solution may be prepared if it is dissolved and used immediately. To prepare the peptide–protein conjugate, add the solution from step 3 to 10 mg of EDC in a test tube. Mix to dissolve. If this ratio of EDC to peptide or protein results in precipitation, scale back the amount of carbodiimide addition until a soluble conjugate is obtained. For some proteins, as little as 0.1 times this amount of EDC may have to be used to maintain solubility.
5. React for 2 hours at room temperature.
6. Purify the conjugate by gel filtration or dialysis using the buffer of choice (for many conjugates 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 is appropriate). If some turbidity has formed during the conjugation procedure, it may be removed by centrifugation or filtration. When using EDC to prepare immunogen conjugates, the presence of some precipitated material is usually not of concern, because precipitated immunogens are often more immunogenic than soluble proteins.

1.2. EDC Plus Sulfo-NHS

The water-soluble carbodiimide EDC may be used to form active ester functionalities with carboxylate groups using the water-soluble compound, NHS (sulfo-NHS) (Thermo Fisher). Sulfo-NHS esters are hydrophilic reactive groups that couple rapidly with amines on target molecules (Staros, 1982; Denney and Blobel, 1984; Kotite *et al*., 1984; Beth *et al*., 1986; Donovan and Jennings, 1986; Jennings and Nicknish, 1985; Ludwig and Jay, 1985; Anjaneyulu and Staros, 1987). Unlike non-sulfonated NHS esters that are relatively water-insoluble and must be first dissolved in organic solvent before being added to aqueous solutions, sulfo-NHS esters typically are water-soluble, longer-lived, and don't hydrolyze quite as quickly in water. However, in the presence of amine nucleophiles that can attack at the carbonyl group of the ester, the sulfo-NHS group rapidly leaves, creating a stable amide linkage with the amine. Sulfhydryl and hydroxyl groups also will react with such active esters, but the products of such reactions, thioesters and esters, are relatively unstable compared to an amide bond.

The advantage of adding sulfo-NHS to EDC reactions is to increase the solubility and stability of the active intermediate, which ultimately reacts with the attacking amine. EDC reacts with a carboxylate group to form an active ester (*o*-acylisourea) leaving group. Unfortunately, this reactive complex is slow to react with amines and can hydrolyze in aqueous solutions, having a rate constant measured in seconds (Hoare and Koshland, 1967). If the target amine does not find the active carboxylate before it hydrolyzes, the desired coupling cannot occur. This is especially a problem when the target molecule is in low concentration compared to water, as in the case of protein molecules. In addition, Nakajima and Ikada (1995) found that if a carboxylate-containing compound can form an anhydride from the *o*-acylisourea intermediate reactive ester, then the yield of amide bond formation is increased. In a similar approach, forming a sulfo-NHS ester intermediate from the reaction of the hydroxyl group on sulfo-NHS with the EDC active-ester complex dramatically increases the resultant amide bond formation. Since the concentration of added sulfo-NHS usually is much greater than the concentration of target molecule, the reaction preferentially proceeds through the more efficient sulfo-NHS ester

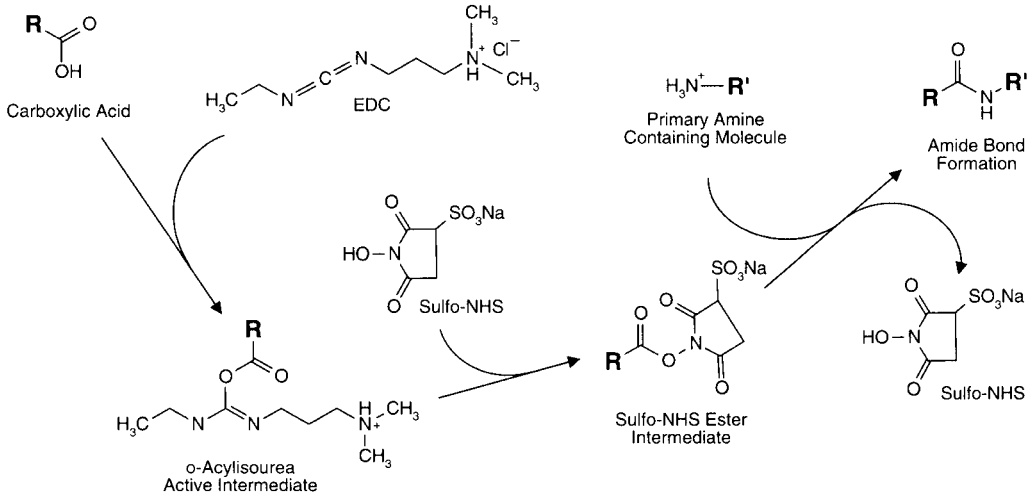


Figure 3.2 The efficiency of an EDC-mediated reaction may be increased through the formation of a sulfo-NHS ester intermediate. The sulfo-NHS ester is more effective at reacting with amine-containing molecules. Thus, higher yields of amide bond formation may be realized using this two-step process as opposed to using a single-step EDC reaction.

intermediate. However, the final product of this two-step reaction is identical to that obtained using EDC alone: the activated carboxylate reacts with an amine to give a stable amide linkage (Figure 3.2).

EDC/sulfo-NHS coupled reactions are highly efficient and usually increase the yield of conjugation significantly over that obtainable solely with EDC. Staros *et al.* (1986) shows that the addition of just 5 mM sulfo-NHS to the EDC coupling of glycine to keyhole limpet hemocyanin increased the yield of derivatization about 20-fold as compared to using EDC alone. This technique also can be used to create activated proteins containing sulfo-NHS esters (Grabarek and Gergely, 1990). A protein can be incubated in the presence of EDC/sulfo-NHS, the active ester form isolated and then mixed with a second protein or other amine-containing molecule for conjugation. This two-step process allows the active species to form only on one protein, thus gaining greater control over the conjugation (Figure 3.3).

In addition to the potential side reactions of EDC as mentioned previously (Section 1.1, this chapter), the additional efficiency obtained by the use of a sulfo-NHS intermediate in the process may cause other problems. In some cases, the conjugation actually may be too efficient to result in a soluble or active complex. Particularly when coupling some peptides to carrier proteins, the use of EDC/sulfo-NHS often causes severe precipitation of the conjugate. Scaling back the amount of EDC/sulfo-NHS added to the reaction may be done to solve this problem. However, eliminating the addition of sulfo-NHS altogether may have to be done in some instances to preserve the solubility of the final product.

The following protocol is a generalized description of how to incorporate sulfo-NHS ester intermediates in EDC conjugation procedures. For specific applications of this technology, the amount of each reagent and unconjugated species may have to be adjusted to obtain an optimal conjugate. See also Chapter 14 and Chapter 9, Section 10 for protocols using EDC/sulfo-NHS in the coupling of proteins to particles and quantum dots, respectively.

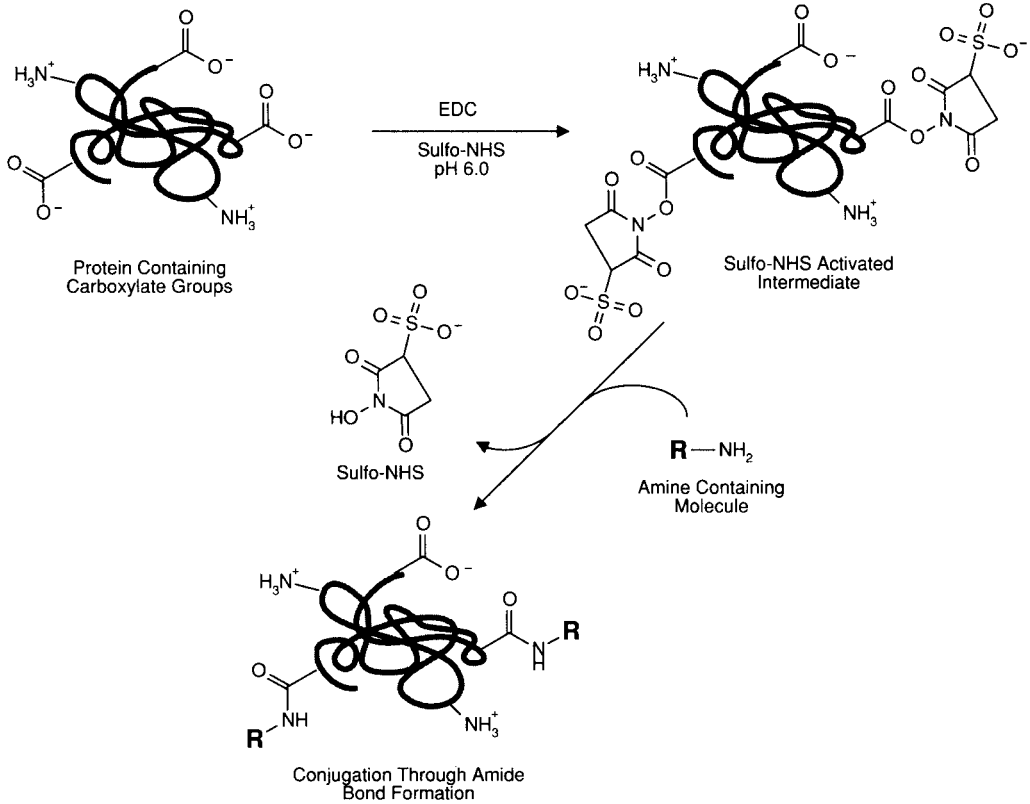


Figure 3.3 EDC may be used in tandem with sulfo-NHS to create an amine-reactive protein derivative containing active ester groups. The activated protein can couple with amine-containing compounds to form amide bond linkages.

Protocol

1. Dissolve the protein to be modified at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, pH 7.4. NaCl may be added to this buffer if desired. For the modification of keyhole limpet hemocyanin (KLH; Thermo Fisher) as described by Staros *et al.*, 1986, include 0.9 M NaCl to maintain the solubility of this high-molecular-weight protein. If lower or higher concentrations of the protein are used, adjust the amounts of the other reactants as necessary to maintain the correct molar ratios.
2. Dissolve the molecule to be coupled in the same buffer used in step 1. For small molecules, add them to the reaction in at least a 10-fold molar excess over the amount of protein present. If possible, the molecule may be added directly to the protein solution in the appropriate excess. Alternatively, dissolve the molecule in the buffer at a higher concentration, and then add an aliquot of this stock solution to the protein solution.
3. Add the solution prepared in step 2 to the protein solution to obtain at least a 10-fold molar excess of small molecule to protein.
4. Add EDC (Thermo Fisher) to the above solution to obtain at least a 10-fold molar excess of EDC over the amount of protein present. Alternatively, a 0.05–0.1 M EDC concentration

in the reaction usually works well. Also, add sulfo-NHS (Thermo Fisher) to the reaction to bring its final concentration to 5 mM. To make it easier to add the correct quantity of EDC or sulfo-NHS, higher concentration stock solutions may be prepared if they are dissolved and used immediately. Mix to dissolve. If this ratio of EDC/sulfo-NHS to peptide or protein results in precipitation, scale back the amount of addition until a soluble conjugate is obtained.
5. React for 2 hours at room temperature.
6. Purify the conjugate by gel filtration or dialysis using the buffer of choice (for many conjugates 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 is appropriate). If some turbidity has formed during the conjugation procedure, it may be removed by centrifugation or filtration.

A modification of a two-step protocol (Grabarek and Gergely, 1990) for the activation of proteins with EDC/sulfo-NHS and subsequent conjugation with amine-containing molecules if given below. The variation in the pH of activation from that described above provides greater stability for the active ester intermediate. At pH 6.0, the amines on the protein will be protonated and therefore be less reactive toward the sulfo-NHS esters that form. In addition, the hydrolysis rate of the esters is dramatically slower at slightly acid pH. Thus, the active species may be isolated in a reasonable time frame without significant loss in conjugation potential. To quench the unreacted EDC, 2-mercaptoethanol is added to form a stable complex with the remaining carbodiimide, according to Carraway and Triplett (1970). In the following protocol, sulfo-NHS is used instead of NHS so that active ester is more water-soluble and ester hydrolysis is slowed (Anjaneyulu and Staros, 1987; Thelen and Deuticke, 1988).

Protocol

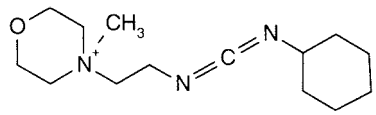
1. Dissolve the protein to be activated in 0.05 M MES, 0.5 M NaCl, pH 6.0 (reaction buffer), at a concentration of 1 mg/ml.
2. Add to the solution in step 1 a quantity of EDC and sulfo-NHS (Thermo Fisher) to obtain a concentration of 2 mM EDC and 5 mM sulfo-NHS. To aid in aliquoting the correct amount of these reagents, they may be quickly dissolved in the reaction buffer at a higher concentration, and then a volume immediately pipetted into the protein solution to obtain the proper molar quantities.
3. Mix and react for 15 minutes at room temperature.
4. Add 2-mercaptoethanol to the reaction solution to obtain a final concentration of 20 mM. Mix and incubate for 10 minutes at room temperature. Note: If the protein being activated is sensitive to this level of 2-mercaptoethanol, instead of quenching the reaction chemically, the activation may be terminated by desalting (step 5).
5. If the reaction was quenched by the addition of 2-mercaptoethanol, the activated protein may be added directly to a second protein or other amine-containing molecule for conjugation. Alternatively, or if no 2-mercaptoethanol was added, the activated protein may be purified from reaction by-products by gel filtration using a desalting resin. The desalting operation should be done rapidly to minimize hydrolysis and recover as much active ester functionality as possible. The use of centrifugal spin columns of some sort may afford the greatest speed in purification (Thermo Fisher). After purification, add the activated protein to the second molecule for conjugation. The second protein or other

amine-containing molecule should be dissolved in 0.1 M sodium phosphate, pH 7.5. This will bring the pH of the coupling medium above pH 7.0 to initiate the active ester reaction.

6. React for at least 2 hours at room temperature.
7. Remove excess reactants by gel filtration or dialysis.

1.3. CMC

CMC, or 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (usually synthesized as the metho *p*-toluene sulfonate salt) (Aldrich), is a water-soluble reagent used to form amide bonds between one molecule containing a carboxylate and a second molecule containing an amine. The presence of the positively charged morpholino group creates its water solubility. Along with EDC (Section 1.1, this chapter), CMC is the only other soluble carbodiimide commonly available for biological conjugations. It was first utilized in peptide synthesis (Sheehan and Hlavka, 1956) and found to be superior to other coupling agents used at the time (Ondetti and Thomas, 1965). It also has been used for the quantitative modification and estimation of total carboxyl groups in protein molecules (Hoare and Koshland, 1967) and for investigating the secondary structure of nucleic acids (Metz and Brown, 1969). Another early application area of CMC, relates not to solution phase crosslinking of two molecules, but to coupling of ligands to insoluble support materials for use in affinity chromatography (Lowe and Dean, 1971; Marcus and Balbinder, 1972; Schmer, 1972).



CMC
1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide
MW 423.58
(as the metho-p-toluene sulfonate salt)

CMC reacts with carboxylate groups by addition of the carboxyl across one of its diimide bonds, resulting in the characteristic active ester, *o*-acylisourea intermediate common to all carbodiimide mechanisms. Nucleophilic attack on this intermediate yields the acylated product—usually an amide bond, resulting from the reaction with a primary amine (Figure 3.4). However, carbodiimide chemistry does create several potential side reactions. Sulfhydryl groups may react with CMC to form a stable covalent complex unreactive toward further conjugation. The reagent also may react with phenols, alcohols, and other nucleophiles to quench the crosslinking reaction. In aqueous solutions, hydrolysis of the carbodiimide and the active ester are by far the most frequent side reactions. Reaction of the ester with water molecules regenerates the carboxylate and releases a soluble isourea by-product.

CMC should be able to participate in the two-step reaction using a sulfo-NHS ester intermediate similar to EDC, however there are no reports in the literature to this effect. Protocols for the use of this reagent in biological crosslinking applications should be essentially the same as those given previously for EDC, except substituting a molar equivalent quantity of CMC. See Sections 1.1 and 1.2 in this chapter for additional information concerning carbodiimide reactions.

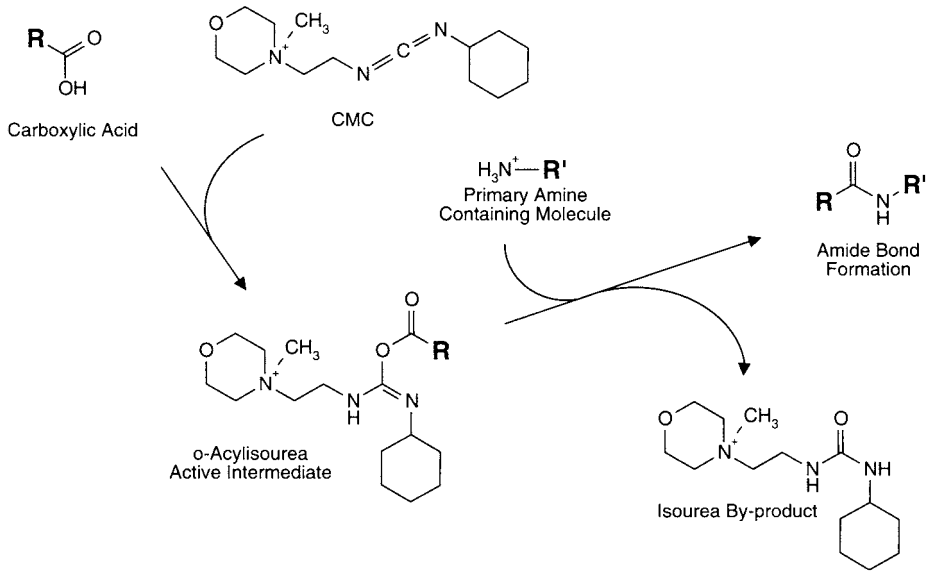
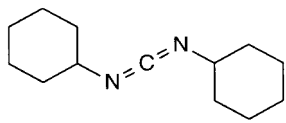


Figure 3.4 The water-soluble carbodiimide CMC reacts with carboxylates to form an active-ester intermediate. In the presence of amine-containing molecules, amide bond formation can take place with release of an isourea by-product.

1.4. DCC

DCC (dicyclohexyl carbodiimide) is one of the most frequently used coupling agents, especially in organic synthesis applications. It has been used for peptide synthesis since 1955 (Sheehan and Hess, 1955) and continues to be a popular choice for creating peptide bonds (Barany and Merrifield, 1980). DCC is water-insoluble, but it has been used in 80 percent DMF for the immobilization of small molecules onto carboxylate-containing chromatography supports for use in affinity separations (Larsson and Mosbach, 1971; Lowe *et al.*, 1973). In addition to forming amide linkages, DCC has been used to prepare active esters of carboxylate-containing compounds using NHS or sulfo-NHS (Staros, 1982). Unlike the EDC/sulfo-NHS reaction described in Section 1.2 (this chapter), active ester synthesis done with DCC is in organic solvent, and therefore doesn't have the hydrolysis problems of water-soluble EDC-formed esters. Thus, DCC is most often used to synthesize active ester containing crosslinking and modifying reagents and not to perform biomolecular conjugations.



DCC
N,N'-Dicyclohexyl
carbodiimide
MW 206.32

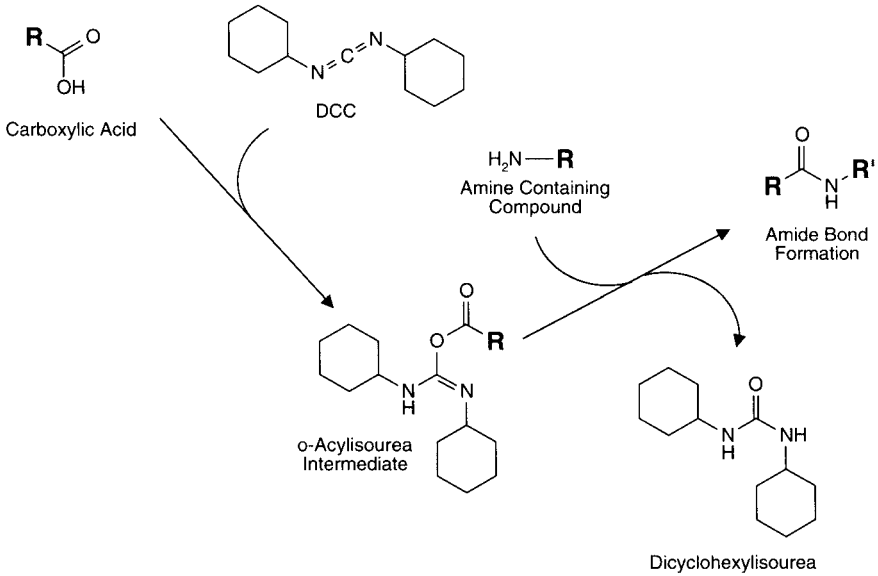


Figure 3.5 The organic-soluble carbodiimide DCC is often used to create amide bonds, especially between water-insoluble compounds.

DCC is a waxy solid that is often difficult to remove from a bottle. Its vapors are extremely hazardous to inhalation and to the eyes. It should always be handled in a fume hood. The isourea by-product of a DCC-initiated reaction, dicyclohexyl urea (DCU) (Figure 3.5), is also water-insoluble and must be removed by organic solvent washing. For synthesis of peptides or affinity supports on insoluble matrices this is not a problem, because washing of the support material can be done without disturbing the conjugate coupled to the support. For solution phase chemistry, however, reaction products must be removed by solvent washings, precipitations, or recrystallizations.

A potential undesirable effect of DCC coupling reactions is the spontaneous rearrangement of the *o*-acylisourea to an inactive *N*-acylurea (Stewart and Young, 1984) (Figure 3.6). The rate of this rearrangement is dramatically increased in aprotic organic solvents, such as DMF.

The activation efficiency of DCC is extraordinarily high, especially in anhydrous solutions that don't have competing hydrolysis problems. *o*-Acylisourea-activated carboxylates may undergo two-side reactions that form other active groups. If DCC is added to an excess of a carboxylate-containing molecule without the presence of an amine-containing target, then the activated carboxylate may react with another carboxylic acid to form a symmetrical anhydride (Figure 3.7). The formation of an anhydride intermediate may be a frequent mechanism in route to the creation of an amide bond with an amine, especially under anhydrous conditions (Rebek and Feitler, 1974; Nakajima and Ikada, 1995). In addition, a DCC-activated carboxylate may react with an amino acid to form an azlactone (Figure 3.8) (Coleman *et al.*, 1990). Both the anhydride and the azlactone will react with amines to form covalent amide linkages. However, the ring-opening reaction of an azlactone will form a different product than the zero-length crosslinking result of coupling directly to an amine-containing molecule (Figure 3.9).

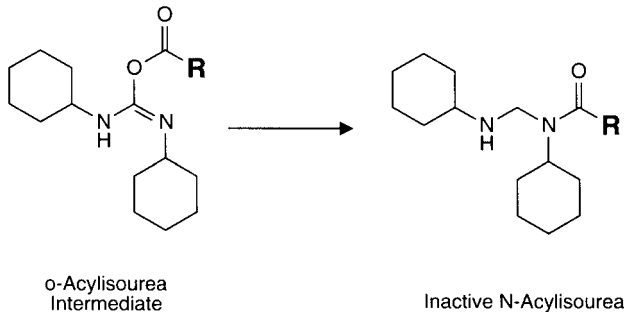


Figure 3.6 The active-ester intermediate formed from the reaction of DCC with a carboxylate group may undergo rearrangement to an inactive *N*-acylisourea product.

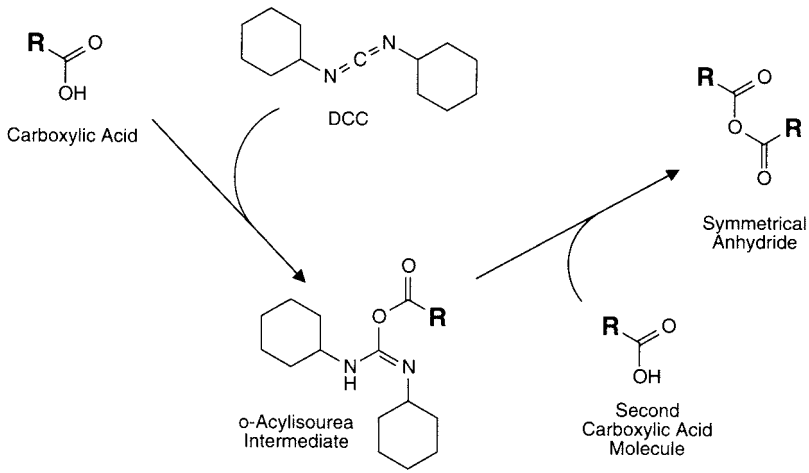


Figure 3.7 The reaction of DCC with a carboxylate compound in excess may create anhydride products in the absence of nucleophiles.

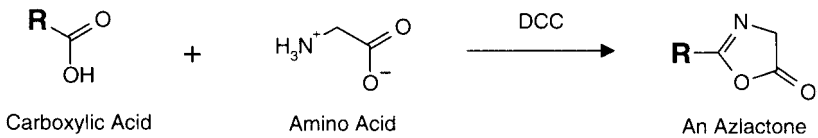


Figure 3.8 A DCC-mediated reaction with a carboxylate group in the presence of a small amino acid may form azlactone rings.

1.5. DIC

DIC (or diisopropyl carbodiimide) is another water-insoluble amide bond-forming agent that has advantages over DCC (Section 1.4, this chapter). It is a liquid at room temperature and

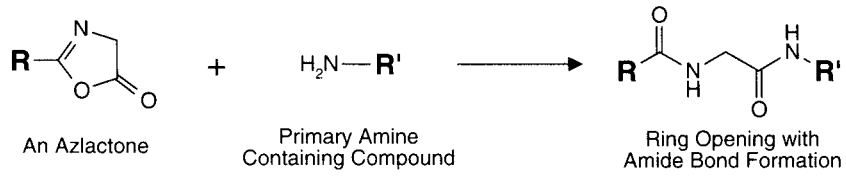


Figure 3.9 An azlactone reacts with amine groups through a ring-opening process, creating amide bond linkages with the attacking nucleophile.

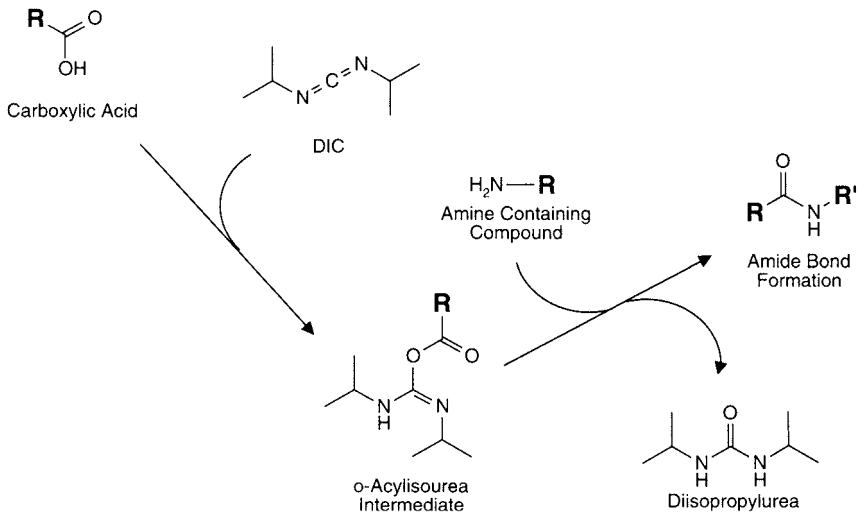
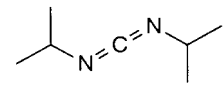


Figure 3.10 The symmetrical carbodiimide DIC reacts with carboxylates to form active-ester intermediates able to couple with amine-containing compounds to form amide bond linkages.

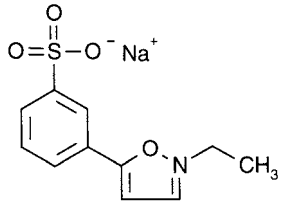
is therefore much easier to dispense than DCC. Its by-products, diisopropylurea and diisopropyl-*N*-acylurea, are more soluble in organic solvents than the DCU by-product of a DCC reaction. DIC reacts similarly to DCC, forming an active *o*-acylisourea intermediate with a carboxylic acid group (Figure 3.10). This active species may then react with a nucleophile such as an amine to form an amide bond. Presumably, all the possible side reactions that DCC may undergo are also possible with DIC, although it is not well documented.



DIC
Diisopropyl carbodiimide
MW 126.2

2. Woodward's Reagent K

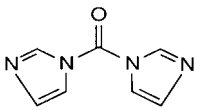
Woodward's reagent K is *N*-ethyl-3-phenylisoxazolium-3′-sulfonate, a zero-length crosslinking agent able to cause the condensation of carboxylates and amines to form amide bonds (Woodward and Olofson, 1961; Woodward *et al*., 1961). The reaction mechanism involved in activating a carboxylate includes the conversion of the reagent under alkaline conditions to a reactive ketoketenimine. This intermediate then reacts with a carboxylate to create an enol ester. The enol ester is highly susceptible to nucleophilic attack. The reaction with an amine proceeds to amide bond formation with loss of the inactive diketo derivative (Figure 3.11). In aqueous solution, the major side reaction is hydrolysis which occurs rapidly (Dunn and Affinsen, 1974). Although Woodward's reagent K has been used successfully for conjugation applications with proteins and other molecules to form amide linkages (Boyer, 1986; Pikuleva and Turko, 1989), its mechanism of reaction was called into question by Johnson and Dekker (1996), who found that the compound reacted with cysteine and histidine groups in *E. coli L*-threonine dehydrogenase, not the available aspartate or glutamate groups. Woodward's reagent K is available from Fluka.



Woodward's Reagent K
N-Ethyl-5-phenylisoxazolium-3'-sulfonate, sodium salt
MW 176

3. *N,N′*-Carbonyldiimidazole

CDI (or *N*,*N*′-carbonyldiimidazole) is a highly active carbonylating agent that contains two acylimidazole leaving groups (Aldrich). The result is that CDI can activate carboxylic acids or hydroxyl groups for conjugation with other nucleophiles, creating either zero-length amide bonds or one-carbon-length *N*-alkyl carbamate linkages between the crosslinked molecules. Carboxylic acid groups react with CDI to form *N*-acylimidazoles of high reactivity. The active intermediate forms in excellent yield due to the driving force created by the liberation of carbon dioxide and imidazole (Anderson, 1958). The active carboxylate then can react with amines to form amide bonds or with hydroxyl groups to form ester linkages (Figure 3.12). Both reaction



CDI
N,N'-Carbonyldiimidazole
MW 162

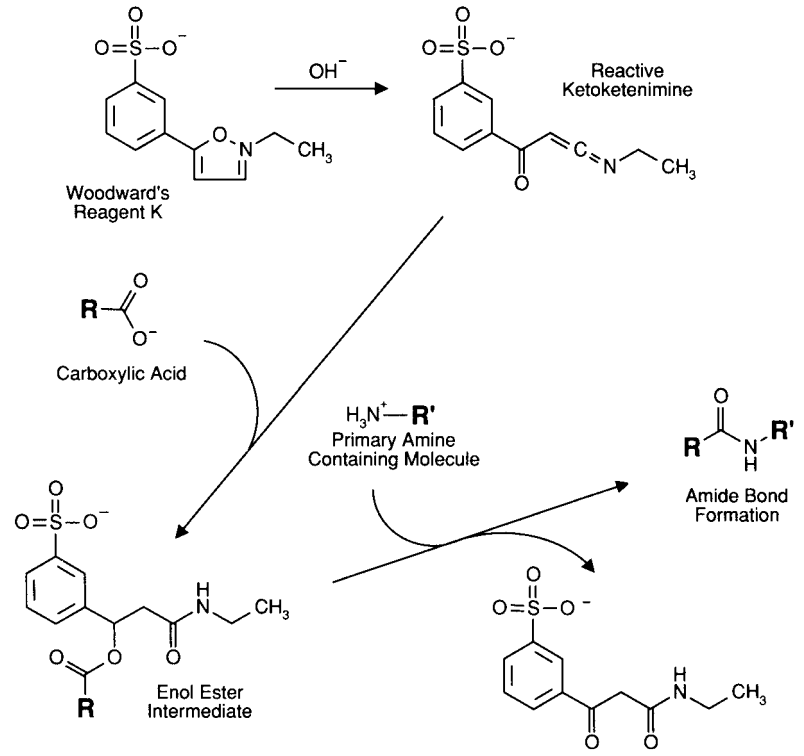


Figure 3.11 Woodward's reagent K undergoes a rearrangement in alkaline solution to form a reactive ketoketenimine. This active species can react with a carboxylate group to create another active group, an enol ester derivative. In the presence of amine nucleophiles, amide bond formation takes place.

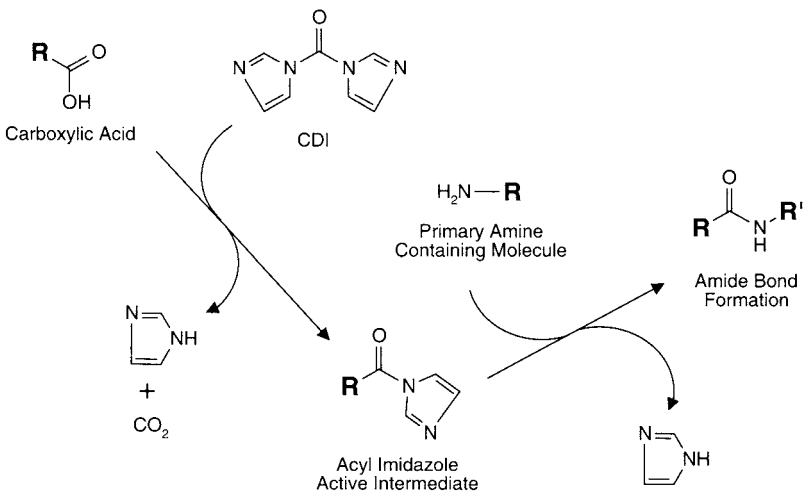


Figure 3.12 CDI reacts with carboxylate groups to form an active acylimidazole intermediate. In the presence of an amine nucleophile, amide bond formation can take place with release of imidazole.

mechanisms have been used successfully in peptide synthesis (Paul and Anderson, 1960, 1962). In addition, activation of a styrene/4-vinylbenzoic acid copolymer with CDI was used to immobilize the enzyme lysozyme through its available amino groups to the carboxyl groups on the matrix (Bartling *et al.*, 1973).

CDI functions as a zero-length crosslinker if the activated species is a carboxylic acid, because the attack of another nucleophile liberates the imidazole leaving group. However, if CDI is used to activate a hydroxyl functional group, the reaction proceeds quite differently. The active intermediate formed by the reaction of CDI with an —OH group is an imidazolyl carbamate (Figure 3.13). Attack by an amine releases the imidazole, but not the carbonyl. Thus, a hydroxyl-containing molecule may be coupled to an amine-containing molecule with the result of a one-carbon spacer, and forming a stable urethane (*N*-alkyl carbamate) linkage. This coupling rocedure has been applied to the activation of hydroxyl-containing chromatography supports for the immobilization of amine-containing affinity ligands (Bethell *et al.*, 1979; Hearn *et al.*, 1979, 1983) and also to the activation of polyethylene glycol for the modification of amine-containing macromolecules (Beauchamp *et al.*, 1983). In addition, Chapter 14 describes the use of CDI for the activation of particles to immobilize proteins or other affinity ligands.

CDI-activated hydroxyls also may undergo a side reaction to form active carbonates. This occurs when an imidazolyl carbamate reacts with another hydroxyl group before the second hydroxyl has had a chance to get activated with CDI. Particularly with adjacent hydroxyls on the same molecule, this can be a problem if a defined reactive species is desired. Any carbonates formed, however, are still reactive toward amines to create carbamate linkages.

Formation of the activated species, whether with a carboxylate or a hydroxyl, must take place in nonaqueous environments due to the rapid breakdown of CDI by hydrolysis. Even in solvents containing small amounts of water, CDI quickly hydrolyzes to CO_2 and imidazole. It is best to use solvents with less than 0.1 percent water to prevent extensive CDI breakdown.

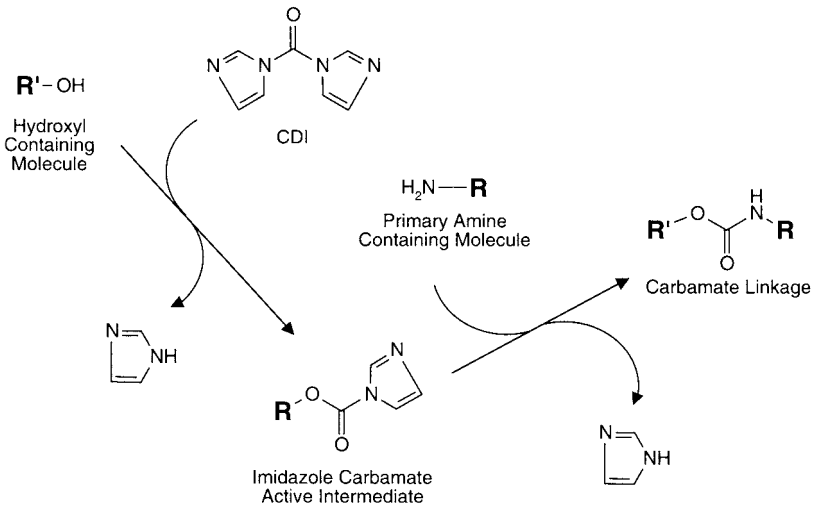


Figure 3.13 CDI reacts with hydroxyl groups to form an active imidazole carbamate intermediate. In the presence of amine-containing compounds, a carbamate linkage is created with loss of imidazole.

Characteristic bubble formation is an indication of reagent hydrolysis, although CO_2 also is released upon reaction with a carboxylic acid. Activation of carboxylates or hydroxyls may be done in dry organic solvents such as acetone, dioxane, DMSO, THF, or DMF. If an excess of CDI is used during the activation step, it should be removed before adding the active intermediate to an amine-containing molecule for conjugation. Alternatively, equal molar quantities of CDI and the molecule to be activated may be mixed to form the active species. After about an hour of activation, add an equivalent molar quantity of the amine-containing target molecule to be conjugated.

Aqueous reaction conditions that result in the best conjugation yields using CDI usually reflect the relative pK_a of the nucleophilic amine being coupled. Proteins are best coupled to CDI-activated supports or molecules in an environment at least one pH unit above their pI values. Frequently the greatest coupling yields occur in alkaline buffers within the range of pH 8.0–10.0. In aqueous solutions, CDI-activated carboxylates or hydroxyls will hydrolyze and slowly lose activity. *N*-Acylimidazoles hydrolyze by loss of imidazole and regenerate the original carboxylate. The imidazole carbamate active species hydrolyzes by loss of CO_2 and imidazole, regenerating in this case, the original hydroxyl group. CDI-activated carboxylic acids hydrolyze faster in aqueous solutions than CDI-activated hydroxyls; however, both experience increasing hydrolysis with increasing pH.

Conjugation reactions using CDI also may be done in organic solutions. This is a distinct advantage if the reactants are not very soluble in aqueous environments. In addition, organic coupling will not result in concomitant loss of activity due to hydrolysis as water-based reactions, thus nonaqueous reactions usually will provide greater yields.

A protocol for the use of CDI in the activation of poly(ethylene glycol) is discussed in Chapter 25, Section 1.4, while CDI activation procedures for particles are described in Chapter 14.

4. Schiff Base Formation and Reductive Amination

Aldehydes and ketones can react with primary and secondary amines to form Schiff bases. A Schiff base is a relatively labile bond that is readily reversed by hydrolysis in aqueous solution. The formation of Schiff bases is enhanced at alkaline pH values, but they are still not completely stable unless reduced to secondary or tertiary amine linkages (Figure 3.14). A number of reducing agents can be used to convert specifically the Schiff base into an alkylamine linkage. Once reduced, the bonds are highly stable. The use of reductive amination to conjugate an aldehyde-containing molecule to an amine-containing molecule results in a zero-length crosslink where no additional spacer atoms are introduced between the molecules.

Reductive amination (or alkylation) may be used to conjugate an aldehyde- or ketone-containing molecule with an amine-containing molecule. The reduction reaction is best facilitated by the use of a reducing agent such as sodium cyanoborohydride, because the specificity of this reagent is toward the Schiff base structure and will not affect the original aldehyde groups. By contrast, sodium borohydride also is used in this reaction, but its strong reducing power rapidly converts any aldehydes not yet reacted into non-reactive hydroxyls, effectively eliminating them from further participation in the conjugation process. Borohydride also may affect the activity of some sensitive proteins, whereas cyanoborohydride is gentler, successfully

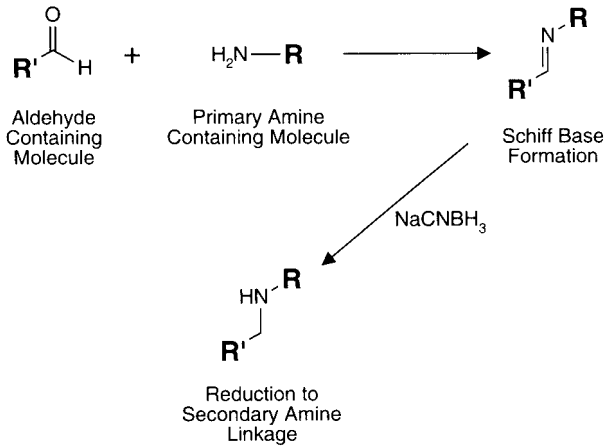


Figure 3.14 Carbonyl groups can react with amine nucleophiles to form reversible Schiff base intermediates. In the presence of a suitable reductant, such as sodium cyanoborohydride, the Schiff base is stabilized to a secondary amine bond.

preserving the activity of even some labile monoclonal a ntibodies. Cyanoborohydride has been shown to be at least 5 times milder than borohydride in reductive amination processes with antibodies (Peng *et al.*, 1987). Other reducing agents that have been explored for reductive amination include various amine boranes and ascorbic acid (Cabacungan *et al.*, 1982; Hornsey *et al.*, 1986).

Immobilization by reductive amination of amine-containing biological molecules onto aldehyde-containing solid supports has been used for quite sometime (Sanderson and Wilson, 1971). The reaction proceeds with excellent efficiency (Domen *et al.*, 1990). The optimum pH for the reaction is alkaline, although good yield can be realized from pH 7 to 10. At the high end of this range (pH 9–10), the formation of the Schiff bases is more efficient, and the yield of conjugation or immobilization reactions can be dramatically increased (Hornsey *et al.*, 1986).

The introduction of aldehyde functional groups into proteins and other molecules can be accomplished by a number of methods (Chapter 1, Section 4.4). Glycoproteins may be oxidized at their carbohydrate residues using sodium periodate or a specific sugar oxidase. Amine groups may be modified to produce a formyl group by reacting with NHS-aldehydes or *p*-nitrophenyl diazopyruvate. The following generalized protocol assumes that the requisite groups are present on the two molecules to be conjugated.

Protocol

1. Dissolve the amine-containing protein to be conjugated at a concentration of 1–10 mg/ml in a buffer having a pH between 7 and 10. Higher pH reactions will result in greater yield of conjugate formation. Suitable buffers include 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2; 0.1 M sodium borate, pH 9.5; or 0.05 M sodium carbonate, 0.1 M sodium citrate, pH 9.5. Avoid amine-containing buffers like Tris.
2. Add a quantity of the aldehyde-containing molecule to the solution in step 1 to obtain the desired molar ratio for conjugation. For instance, if the amine-containing protein is

an antibody and the aldehyde-containing protein is an enzyme such as horseradish peroxidase (HRP), a typical molar ratio for the reaction might be 2–4 moles of HRP per mole of antibody.

3. Add 10 µl of 5 M sodium cyanoborohydride in 1 N NaOH (Aldrich) per ml of the conjugation solution volume. *Caution: Highly toxic compound. Use a fume hood and be careful to avoid skin contact with this reagent.*
4. React for 2 hours at room temperature.
5. To block unreacted aldehyde sites, add 20 µl of 3 M ethanolamine (pH adjusted to desired value with HCl) per ml of the conjugation solution volume. React for 15 minutes at room temperature.
6. Purify the conjugate by dialysis or gel filtration using a buffer suitable for the nature of the proteins being crosslinked.

4

Homobifunctional Crosslinkers

The first crosslinking reagents used for modification and conjugation of macromolecules consisted of bireactive compounds containing the same functionality at both ends (Hartman and Wold, 1966). Most of these homobifunctional reagents were symmetrical in design with a carbon chain spacer connecting the two identical reactive ends (Figure 4.1). Like molecular rope, these reagents could tie one protein to another by covalently reacting with the same common groups on both molecules. Thus, the lysine ε-amines or N-terminal amines of one protein could be crosslinked to the same functionalities on a second protein simply by mixing the two together in the presence of the homobifunctional reagent.

The ability to so easily link two proteins or other molecules having different binding specificities or catalytic activities opened the potential for creating a new universe of unique and powerful reagent systems for use in assay and targeting applications. The variety and reactivity of homobifunctional reagents multiplied dramatically throughout the 1970s and 1980s. Today, there are dozens of commercially available crosslinkers possessing almost every length and reactivity desired.

The main disadvantage, however, of using simple homobifunctional reagents is the potential for creating a broad range of poorly defined conjugates (Avrameas, 1969). When crosslinking two proteins, for example, the reagent may react initially with either one of the proteins, forming an active intermediate. This activated protein may form crosslinks with the second protein or react another molecule of the same type. It also may react intramolecularly with other functional

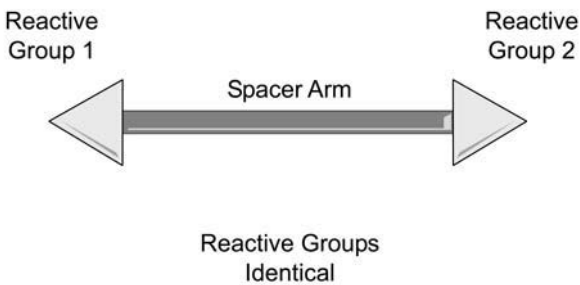


Figure 4.1 The general design of a homobifunctional crosslinking agent. The two reactive groups are identical and typically are located at the ends of an organic spacer arm. The length of the spacer may be designed to accommodate the optimal distance between two molecules to be conjugated.

groups on part of its own polypeptide chain. In addition, other crosslinking molecules may continue to react with these intermediates to form various mixed oligomers, including severely polymerized products that may even precipitate (see Chapter 1, Section 1.2).

The problem of poorly defined conjugation products is exacerbated in single-step reaction procedures using homobifunctional reagents. Single-step procedures involve the addition of all reagents at the same time to the reaction mixture. This technique provides the least control over the crosslinking process and invariably leads to a multitude of products, only a small percentage of which represent the desired conjugate. Excessive conjugation may cause the formation of insoluble complexes that consist of very high-molecular-weight polymers. For example, one-step glutaraldehyde conjugation of antibodies and enzymes (Chapter 20, Section 1.2) often results in significant oligomers and precipitated conjugates. To overcome this shortcoming, two-step reaction procedures have been developed using homobifunctional reagents. Controlled, two-step conjugation protocols somewhat alleviate the polymerization problem with homobifunctional reagents, but can never totally avoid it.

In two-step protocols, one of the proteins to be conjugated is reacted with the homobifunctional reagent and excess crosslinker and by-products are removed. In the second stage, the activated protein is mixed with the other protein or molecule to be conjugated, and the final conjugation process occurs (Figure 4.2).

One potential problem of such two-step procedures is hydrolysis of the activated intermediate before addition of the second molecule to be conjugated. For instance, *N*-hydroxysuccinimide (NHS) ester homobifunctionals hydrolyze rapidly and may degrade before the second stage of the crosslinking is initiated. In addition, the use of homobifunctional reagents in two-step protocols still produces many of the problems associated with single-step procedures, because the first protein can crosslink and polymerize with itself long before the second protein is added. Since the first protein to be activated has target functional groups on every molecule that can couple with both reactive groups of the crosslinker, both ends of the reagent potentially can react. This inherent capacity to polymerize uncontrollably unfortunately is characteristic of all homobifunctional reagents, even in multi-step protocols.

Although their shortcomings in this regard are clearly recognized, homobifunctional reagents continue to be popular choices for all kinds of conjugation applications. The fact is, in many crosslinking functions, they work well enough to form effective conjugates. Even glutaraldehyde-mediated antibody–enzyme conjugates still are commonly utilized in everything from research to diagnostics.

The particular crosslinkers discussed in this section are the types most often referred to in the literature or are commercially available. Many other forms of homobifunctional reagents containing almost every conceivable chain length and reactivity can be found mentioned in the scientific literature.

1. Homobifunctional NHS Esters

Carboxylate groups activated with NHS esters are highly reactive toward amine nucleophiles. In the mid-1970s, NHS esters were introduced as reactive ends of homobifunctional crosslinkers (Bragg and Hou, 1975; Lomant and Fairbanks, 1976). Their excellent reactivity at physiological pH quickly established NHS esters as viable alternatives to the imidoesters predominating at the time (Section 2, this chapter).

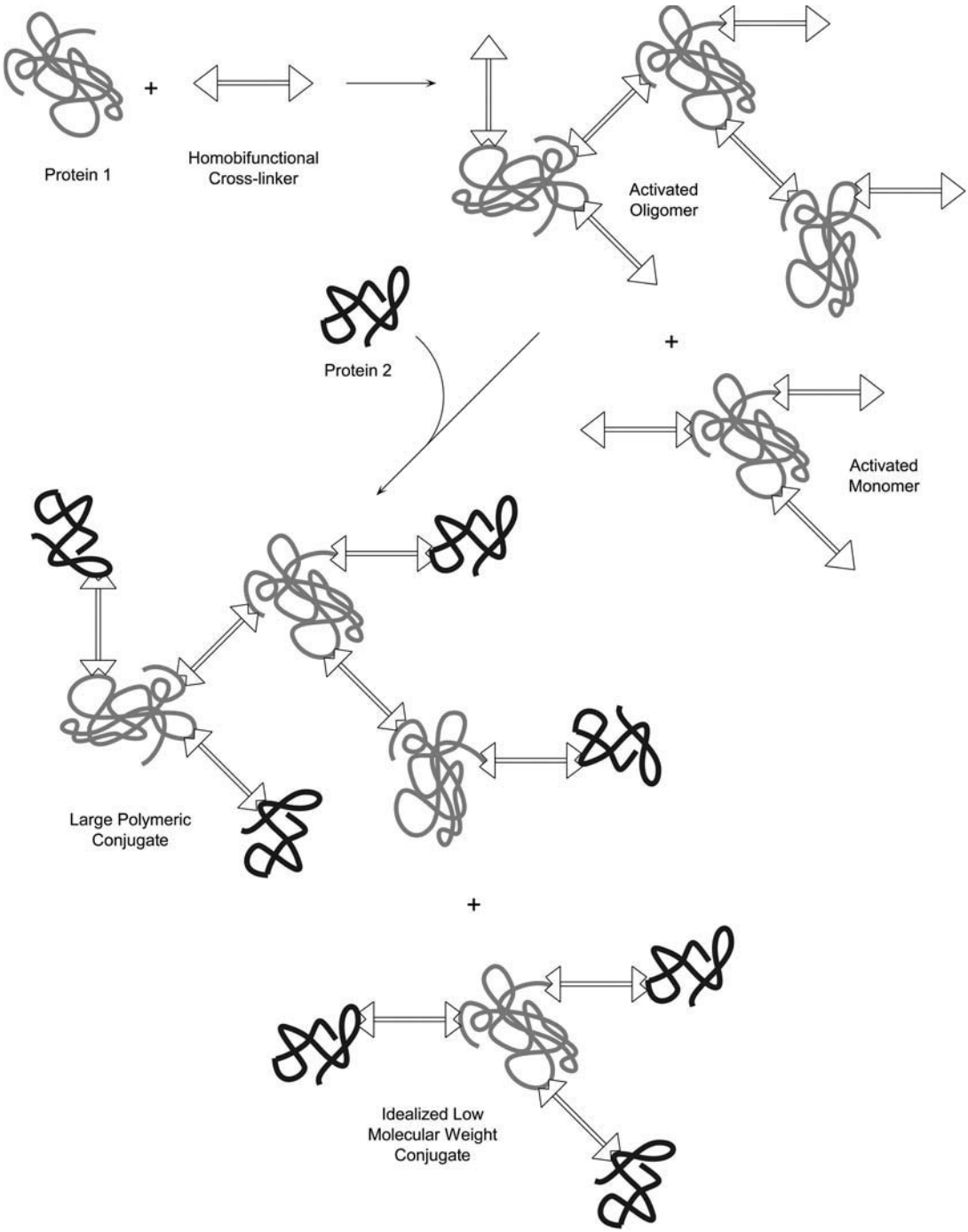


Figure 4.2 Homobifunctional crosslinkers may be used in a two-step process to conjugate two proteins or other molecules. In the first step, one of the two proteins is reacted with the crosslinker in excess to create an active intermediate. After removal of remaining crosslinker, a second protein is added to effect the final conjugate. Two-step reaction schemes somewhat limit the degree of polymerization obtained when using homobifunctional reagents, but can't entirely prevent it.

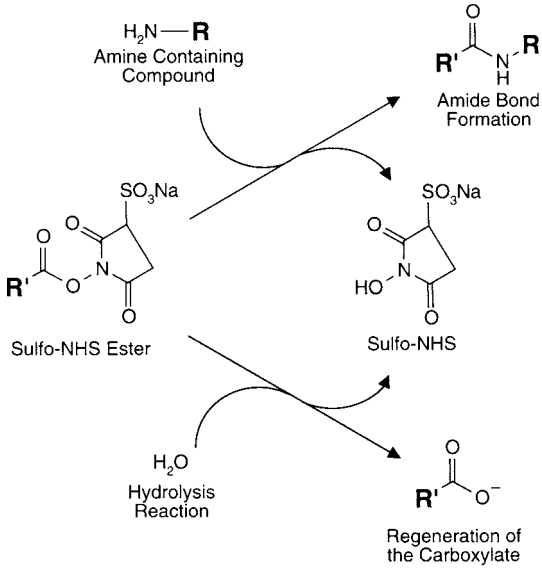


Figure 4.3 In aqueous solution, a sulfo-NHS ester can either couple to an amine group to form an amide bond or react with water to hydrolyze back to a carboxylate. Both processes release the sulfo-NHS leaving group.

Unfortunately, many NHS ester-containing crosslinkers are insoluble in aqueous buffers. Most protocols involve dissolving the compound at a relatively high concentration in an organic solvent and aliquoting the required quantity into the reaction medium. Prior dissolution helps to maintain at least some solubility in the buffered crosslinking environment. Most of the time, however, the addition of an organic-solubilized crosslinker into a buffered solution results in a microprecipitate that slowly goes into solution during the course of reaction.

In the early 1980s, Staros prepared a derivative of NHS that aids in the water solubility of NHS ester crosslinkers (Staros, 1982). *N*-hydroxysulfosuccinimide (sulfo-NHS) esters possess a negatively charged sulfonate group on carbon number 2 or 3 of the succinimide ring (Figure 4.3). Crosslinking reagents-containing sulfo-NHS esters have half-lives of hydrolysis on the order of hours, sometimes even better than their NHS ester analogs (Anjaneyulu and Staros, 1987). The sulfo-NHS ester often lends enough charge and polarity to a crosslinker to provide water solubility and thus eliminate the need for organic solvent dissolution. In addition, sulfo-NHS crosslinkers may be used for surface only modification of membranes and cells, since they are more hydrophilic and will not penetrate the lipid environment of the membrane (Staros, 1982, 1988; Staros *et al.*, 1987). By contrast, many of the more hydrophobic NHS ester crosslinkers can be used to traverse the cell membrane and modify intracellular components.

NHS or sulfo-NHS ester-containing homobifunctional crosslinkers react with nucleophiles to release the NHS or sulfo-NHS leaving group and form an acylated product. The reaction of such esters with a sulfhydryl or hydroxyl group is possible, but doesn't yield stable conjugates, forming thioesters and ester linkages which may hydrolyze in aqueous environments. Histidine side chain nitrogens of the imidazolyl ring also may be acylated with an NHS ester reagent, but they too hydrolyze rapidly (Cuatrecasas and Parikh, 1972). Reaction with primary and secondary amines, however, creates stable amide and imide linkages, respectively, that don't readily

break down. In protein molecules, NHS ester crosslinking reagents primarily react with the α-amines at the N-terminals and the abundant ε-amines of lysine side chains.

NHS ester crosslinking reactions in aqueous solutions consist of the potential for hydrolysis as well as the desired amide bond formation. Crosslinkers-containing NHS esters have fairly good stability in aqueous solutions, despite their susceptibility to attack and breakdown by water. Studies done on the NHS ester-containing homobifunctional reagent, dithio*bis*(succini midylpropionate) (DSP), indicate that the activated carboxylates have half-lives on the order of hours at physiological pH. However, hydrolysis and amine reactivity both increase with increasing pH. At 0°C at pH 7.0, the half-life of the crosslinking reagent DSP is 4–5 hours (Lomant and Fairbanks, 1976). At pH 8.0 at 25°C it falls to about 1 hour (Staros, 1988), and at pH 8.6 and 4°C the half-life is only 10 minutes (Cuatrecasas and Parikh, 1972).

The rate of hydrolysis may be monitored by measuring the increase in absorptivity at 260 nm as the NHS leaving group is cleaved. The molar extinction coefficient of the NHS group in solution is $8.2 \times 10^3\,M^{-1}cm^{-1}$ in Tris buffer at pH 9.0 (Carlsson *et al.*, 1978), but somewhat decreases to $7.5 \times 10^3\,M^{-1}cm^{-1}$ in potassium phosphate buffer at pH 6.5 (Partis *et al.*, 1983). Unfortunately, the sensitivity of this absorptivity usually does not allow for measuring the rate of reaction in an actual crosslinking procedure.

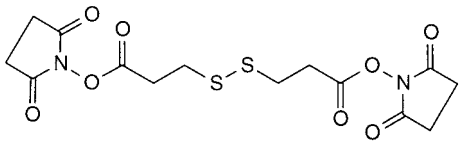
To maximize the modification of amines and minimize the effects of hydrolysis, maintain a high concentration of protein or other target molecule. By adjusting the molar ratio of crosslinker to target molecule(s), the level of modification and conjugation may be controlled to create an optimal product.

The reaction buffer chosen for the conjugation reaction should be free of extraneous amines. Avoid Tris or glycine buffers. Also, avoid imidazole buffers, since the nitrogens of the imidazole ring may react with the active ester and then quickly hydrolyze. The effect is that imidazole only acts to catalyze the hydrolysis process. The pH of the reaction should be in the range of 7–9 to promote the unprotonated state of primary amines, which is the nucleophilic species that most effectively attacks the activated carbonyl group. Dissolve NHS ester crosslinkers that are insoluble in water in an organic solvent such as DMF or DMSO prior to addition to the reaction medium. Sulfo-NHS crosslinkers may be added directly to the reaction mixture or pre-dissolved in buffer at a higher concentration before adding an aliquot to the reaction. Aqueous stock solutions should be used immediately to prevent extensive hydrolysis of the active esters.

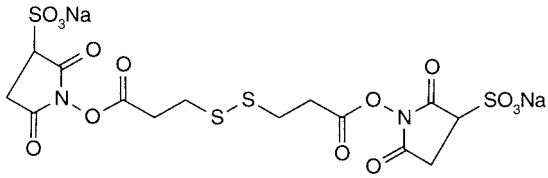
NHS ester crosslinking reagents also may be used in organic solvent-based reactions without the competing hydrolysis problem provided the target molecules are soluble and stable in such environments. In this case, both molecules to be conjugated must be soluble in the solvent. DMF, DMSO, acetone, and dioxane are examples of solvents that can be utilized as the reaction medium. Refer to any published solubility data on the crosslinking reagent of choice to see which solvents are most appropriate.

1.1. DSP and DTSSP

Lomant's reagent [dithiobis(succinimidylpropionate), DSP] is a homobifunctional NHS ester crosslinking agent containing an 8-atom spacer of 12 Å in length (Lomant and Fairbanks, 1976) (Thermo Fisher). It is symmetrically constructed around a central disulfide group that is cleavable after conjugation with typical disulfide reducing agents (Chapter 1, Section 4.1).



DSP
Dithiobis(succinimidylpropionate)
MW 404.42
12 Å



DTSSP
3,3'-Dithio*bis*(sulfosuccinimidylpropionate)
Water Soluble
MW 608.51
12 Å

DSP is water-insoluble and must be pre-dissolved in an organic solvent before addition to a conjugation reaction. Concentrated stock solutions may be prepared in DMF or DMSO and an aliquot added to a buffered reaction medium. The NHS ester reaction occurs most efficiently at pH 7–9, with hydrolysis of the active species accelerating the greater the pH. The crosslinking buffers should be free of amine-containing components other than the target molecules to be conjugated. Avoid Tris or glycine buffers. A reaction buffer consisting of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5 works well for most applications involving the crosslinking of two purified proteins. The relatively high concentration of sodium phosphate is used to prevent pH drift downward during the course of the reaction. For *in vitro* crosslinking of cellular components such as membrane proteins, a more dilute PBS buffer-containing isotonic saline is more appropriate. Since DSP is a hydrophobic reagent, it is able to penetrate the cell membrane and conjugate membrane components. For this reason, it has become quite popular for use in investigating the interactions of membrane proteins.

DSP reacts with ε-amine groups on the side chains of lysine residues or the α-amine at the N-terminal of proteins to form amide linkages. Amine-containing macromolecules may be reversibly crosslinked with this reagent and later cleaved with dithiothreitol (DTT) or 2-mercaptoethanol (Figure 4.4). For reductive cleavage of conjugated molecules, add 10–50 mM DTT, and incubate at 37°C for 30 minutes. Alternatively, the conjugate may be reduced prior to electrophoresis using SDS sample buffer with 5 percent 2-mercaptoethanol at elevated temperatures.

Lomant's reagent is one of the most popular of all crosslinking agents, especially for the investigation of protein interactions. Hordern *et al.* (1979) used it to investigate the spatial relationships in the capsid polypeptides of the mengo virion. It has been used to study the interactions of proteins involved with active transport (dePont *et al.*, 1980; Joshi and Burrows, 1990), identifying crosslinks involving cytochrome P-450 (Baskin and Yang, 1982), characterization of cell surface receptors for colony-stimulating factor (Park *et al.*, 1986), the determination of various membrane antigens by crosslinking to specific monoclonal antibodies (Hamada and Tsuro, 1987), studying prothrombin self-association (Tarvers *et al.*, 1982), investigating

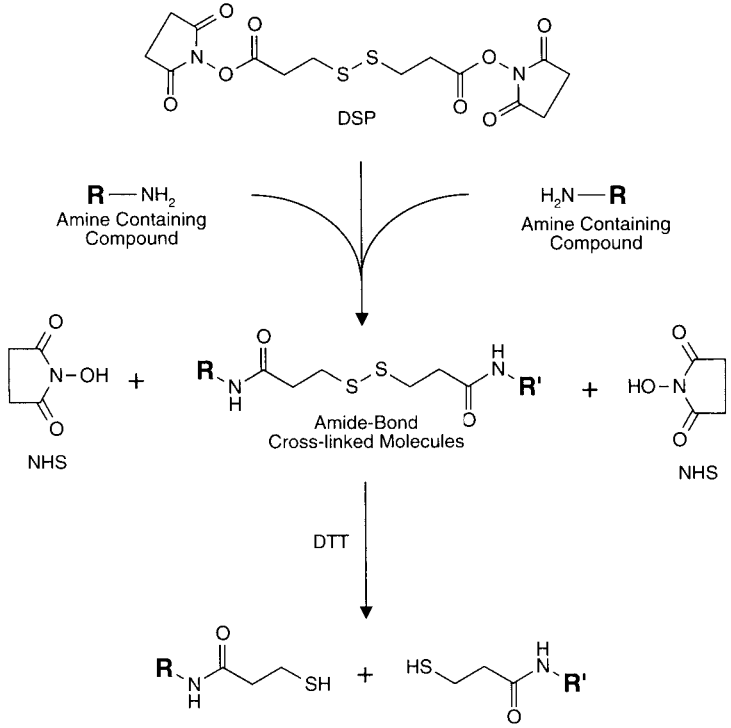


Figure 4.4 The reaction of DSP with amine-containing molecules yields amide bond crosslinks. The conjugates may be cleaved by reduction of the disulfide bond in the cross-bridge with DTT.

chemotaxis in *E. coli* (Chelsky and Dahlquist, 1980), molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium (Laburthe *et al.*, 1984), in studying the crosslinking of the affinity purified CCAAT transcription, α-CP1 (Kim and Sheffrey, 1990), and to fix tissue for immunostaining procedures (Xiang *et al.*, 2004). The dithiol bridge in DSP also has been used to activate metal surfaces or metal particles through dative bonding (Grubor *et al.*, 2004). The presence of the disulfide group creates two dative bonds in each linker, which significantly increases the stability of the surface bonds. DSPs two NHS ester groups then can be used to couple amine-containing molecules to the metallic surface, see Chapter 2, Section 2.8 for additional information.

The sulfo-NHS version of DSP, dithio*bis*(sulfosuccinimidyl propionate) or DTSSP, is a water-soluble analog of Lomant's reagent that can be added directly to aqueous reactions without prior organic solvent dissolution (Staros, 1982). DTSSP still contains the disulfide center portion that is cleavable with the proper reducing agents, and the sulfo-NHS ends have virtually the same reactivity as DSP (Figure 4.5). Due to its hydrophilicity, however, DTSSP will not penetrate cellular membranes as does its more hydrophobic analog, DSP. It is therefore an excellent choice for the crosslinking of cell surface components without affecting intracellular substances.

DTSSP reportedly has been used to crosslink the extracytoplasmic domain of the anion exchange channel in human erythrocytes (Staros and Kakkad, 1983), for the characterization

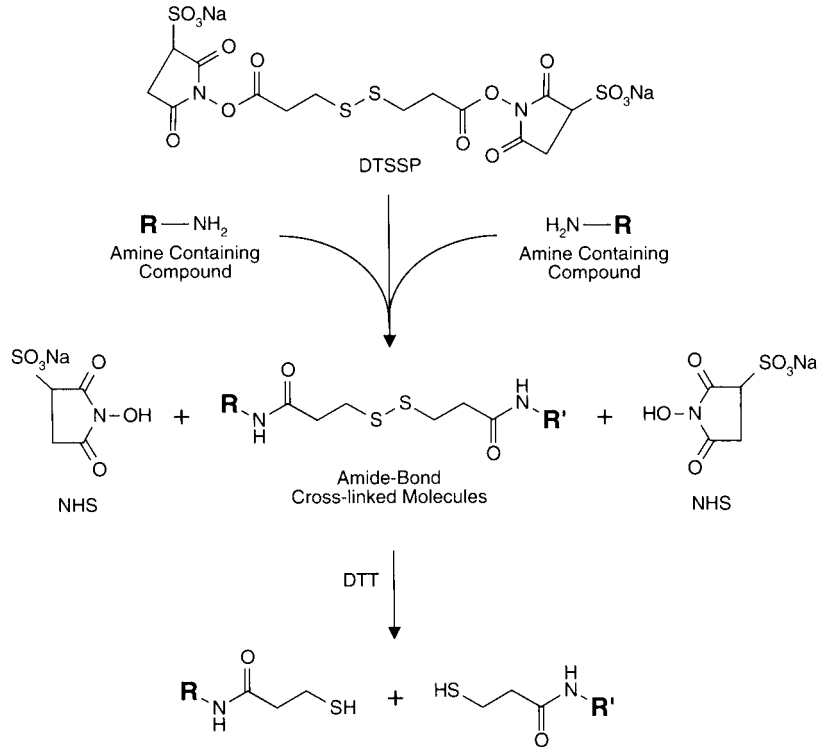


Figure 4.5 DTSSP can form crosslinks between two amine-containing molecules through amide linkages. The conjugates may be cleaved by disulfide reduction using DTT.

of a ribosomal complex in *B. subtilis* (Caufield *et al.*, 1984), to investigate ascites hepatoma cytokeratin filaments (Knoller *et al.*, 1991), for the study of the B-lymphocyte Fc receptor for IgE (Waugh *et al.*, 1989), and to crosslink platelet glycoproteins (Jung and Moroi, 1983).

1.2. DSS and BS[3]

Disuccinimidyl suberate (DSS) is an amine reactive, homobifunctional, NHS ester, crosslinking reagent that produces an 8-atom bridge (11.4 Å) between conjugated molecules (Figure 4.6) (Thermo Fisher). Its hydrocarbon chain is non-cleavable, so crosslinks formed are irreversible. Many of the reported applications of DSS involve investigations of receptor–ligand binding on cell surfaces using radiolabeled molecules. The crosslinker is hydrophobic and must be solubilized in organic solvent prior to addition to a conjugation reaction. Pre-dissolving in dry dioxane, DMF, or DMSO may be done at higher concentration and then an aliquot added to the aqueous reaction medium as needed. The final concentration of the organic solvent in the buffered reaction should not exceed 10 percent to avoid precipitation of biomolecules. Stock solutions should be prepared fresh. DSS is membrane permeable and is therefore useful for intracellular and intramembrane conjugations. The optimum conditions for the crosslinking

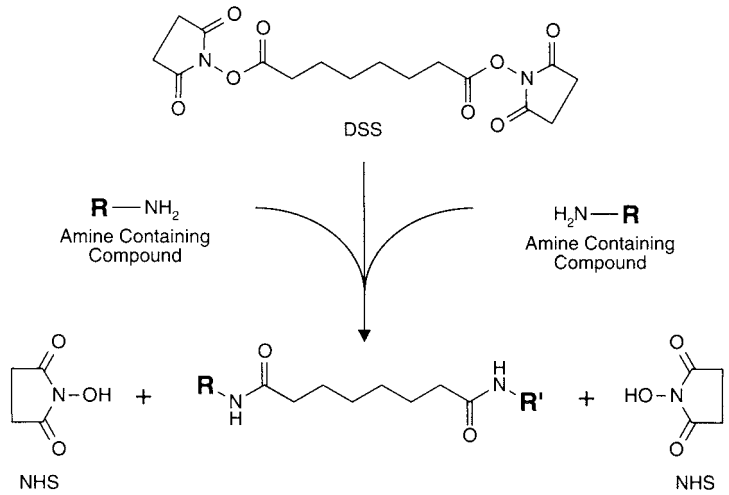
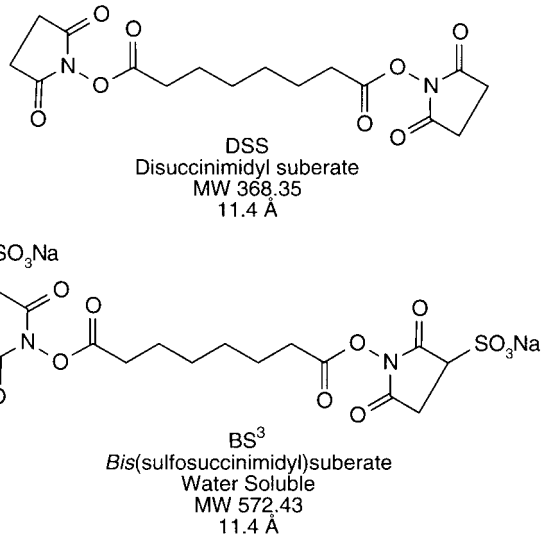


Figure 4.6 DSS reacts with two amine-containing molecules to form amide bond crosslinks. The cross-bridge is non-cleavable.

reaction are a pH range of 7–9 using buffers and other salts which contain no amines. Avoid the use of Tris or glycine. A phosphate buffer (PBS) at physiological pH works well, see Section 1 (this chapter) for additional information on NHS ester reactions.



Reported applications of DSS include crosslinking the A and B subunits of ricin (Montesano *et al.*, 1982), studying human somatotropin and the components of the lactogenic-binding sites of rat liver (Caamano *et al.*, 1983), crosslinking CSF-1 to its cell-surface receptor (Morgan

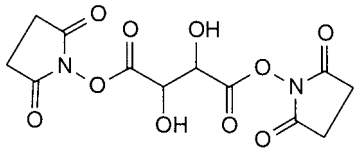
and Stanley, 1984), studying angiotensin II interactions with its receptor (Petruzzelli *et al*., 1985), crosslinking of vasoactive intestinal peptide to its receptor on human lymphoblasts (Wood and O'Dorisio, 1985), investigating insulin-dependent protein kinases (Petruzzelli *et al*., 1985), identifying a cellular receptor for tumor necrosis factor (TNF) (Kull *et al*., 1985), affinity crosslinking of atrial natriuretic factor in aorta membranes (Vandelen *et al*., 1985), studying the receptor for human interferon (Rashidbaigi *et al*., 1986), crosslinking of endorphin to membranes rich in opioid receptors (Helmeste *et al*., 1986), immunoprecipitation studies of the crosslinked complex of parathyroid hormone with its receptor (Wright *et al*., 1987), binding of human interferon Y to its receptor (Novick *et al*., 1987), identifying the erythropoietin receptor on Friend virus-infected erythroid cells (Sawyer *et al*., 1987), and binding of the p75 peptide to an interleukin 2 receptor (Tsudo *et al*., 1987).

Bis(sulfosuccinimidyl) subsrate (BS^3) is an analog of DSS that contains sulfo-NHS esters on both carboxylates. The affect of the negative charges provided by the sulfonate groups lends water solubility to the compound. Prior organic solvent dissolution (before addition to a reaction) is not necessary. The hydrophilicity of BS^3 also makes it membrane impermeable. Therefore, cell labeling with BS^3 results in hydrophilic-region modification and crosslinking, targeting surface functionalities, whereas DSS is capable of targeting hydrophobic regions within the membrane structure itself. As with DSS, BS^3 is non-cleavable, and thus all crosslinks formed are irreversible. The reactivity of the sulfo-NHS esters is identical to NHS esters, being highly reactive toward amines in the pH range of 7–9.

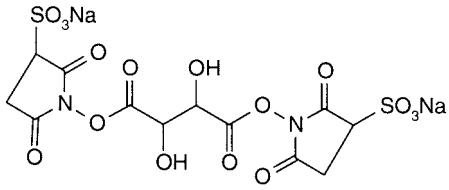
Reported applications of BS^3 include crosslinking of the β-endorphin–calmodulin interaction (Staros, 1982), crosslinking of the extracellular domain of intact human erythrocytes' anion exchange channel (Staros and Kakkad, 1983), crosslinking of hepatoma cytokeratin filaments (Ward *et al*., 1985), investigating the β-lymphocyte Fc receptor for IgE (Lee and Conrad, 1985; Staros *et al*., 1987), crosslinking of the tri-peptide Arg–gly–asp to an adhesion receptor on platelets (Souza *et al*., 1988), crosslinking of the large and small subunits of cytochrome b559 (Knoller *et al*., 1991), and for general receptor–ligand crosslinking (Waugh *et al*., 1989). See also Dihazi and Sinz (2003), Koller *et al*. (2004), and Law *et al*. (2002) for additional applications of BS^3 in proteomic applications. Also see Ishmael *et al*. (2005) and Longshaw *et al*. (2004) for additional applications involving DSS in studying protein interactions.

1.3. DST and Sulfo-DST

Disuccinimidyl tartarate (DST) is a homobifunctional NHS ester crosslinking reagent that contains a central diol that is susceptible to cleavage with sodium periodate (Thermo Fisher). DST forms amide linkages with α-amines and ε-amines of proteins or other amine-containing molecules (Figure 4.7). The reagent is fairly insoluble in aqueous buffers, but it may be predissolved in THF, DMF, or DMSO prior to addition of an aliquot to a reaction. Optimal conditions for reactivity include a pH range of 7–9 with no extraneous amines present that may cross-react with the NHS esters. Avoid Tris, glycine, or imidazole buffers. Subsequent to conjugating proteins with DST, the crosslinks may be broken for analysis by treatment with 0.015 M sodium periodate.



DST
Disuccinimidyl tartarate
MW 344.24
6.4 Å



Sulfo-DST
Disulfosuccinimidyl tartarate
Water Soluble
MW 548.34
6.4 Å

Reported applications of DST include the crosslinking of ubiquinone cytochrome C reductase (Smith *et al.*, 1978), characterization of the cell surface receptor for colony-stimulating factor (Park *et al.*, 1986), investigation of the Ca^{+2}-, Mg^{+2}-activated ATP of *E. coli* (Bragg and Hou, 1980), and characterization of human properdin polymers (Farries and Atkinson, 1989).

Disulfosuccinimidyl tartarate (sulfo-DST) is an analog of DST that contains sulfo-NHS esters. The negatively charged sulfonate groups contribute enough hydrophilicity to provide water solubility for the reagent without the need for organic solvent dissolution before adding it to a crosslinking reaction. The conditions for conjugation are otherwise identical to DST. DST and sulfo-DST also have been used to study protein–lipid complexes (Predescu *et al.*, 2001), to investigate the binding protein of corticotropin-releasing factor (Jahn *et al.*, 2002), and the acidic C-terminal domain of rna1p (Haberland *et al.*, 1997).

1.4. BSOCOES and Sulfo-BSOCOES

BSOCOES [*bis*[2-(succinimidyloxycarbonyloxy)ethyl]sulfone] is a water-insoluble, homobifunctional NHS ester crosslinking reagent that contains a central sulfone group, which is cleavable under alkaline conditions (Figure 4.8) (Thermo Fisher). The two NHS ester ends are reactive with amine groups in proteins and other molecules to form stable amide linkages. Once proteins are crosslinked using this reagent, they may be dissociated for analysis by raising the pH to 11.6 and incubating for 2 hours at 37°C. The sulfone group is base labile under these conditions, and the conjugate cleaves at the center of the bridge.

BSOCOES is a hydrophobic crosslinker and therefore must be dissolved in organic solvent prior to its addition to an aqueous reaction medium. Preparing a stock solution in DMF or DMSO and then adding an aliquot to the crosslinking reaction is recommended. Do not exceed

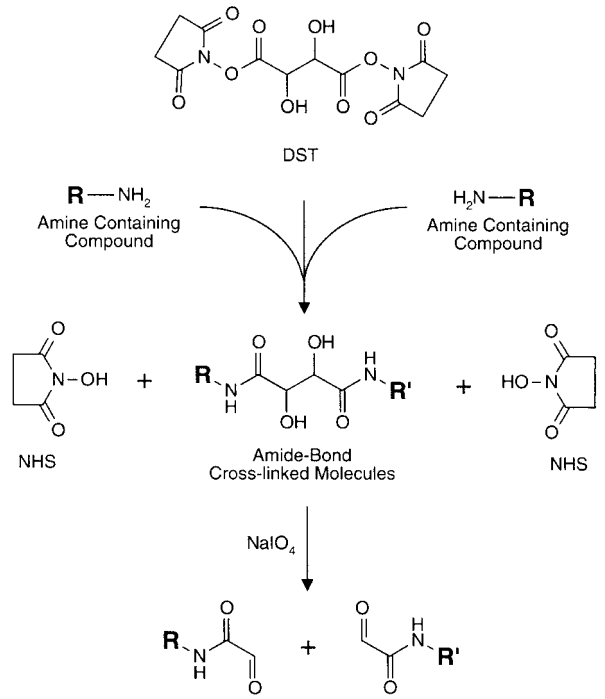


Figure 4.7 DST may be used to crosslink amine-containing molecules, forming amide bond linkages. The central diol of the cross-bridge is cleavable by treatment with sodium periodate.

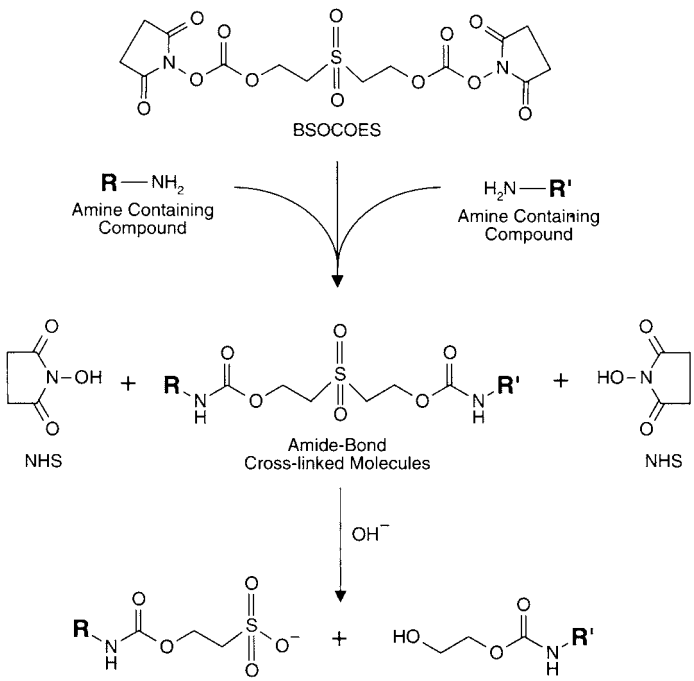
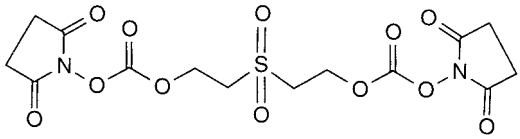
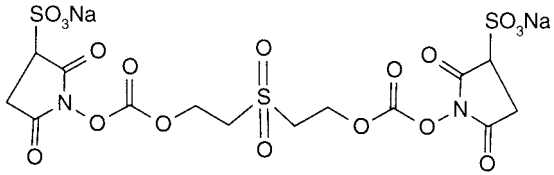


Figure 4.8 BSOCOES reacts with amine-containing molecules to create amide bond crosslinks. The internal sulfone group is cleavable under alkaline conditions.

a concentration of more than 10 percent organic solvent in the buffered reaction to avoid protein precipitation.



BSOCOES
Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone
MW 436.36
13 Å



Sulfo-BSOCOES
Bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone
Water Soluble
MW 640.46
13 Å

Reported applications of BSOCOES include studying the polypeptide antigens on lymphocyte cell surfaces (Zarling *et al.*, 1980), crosslinking labeled β-endorphin to its opioid receptors (Howard *et al.*, 1985), and isolation and characterization of calcitonin receptors in rat kidney (Bouizar *et al.*, 1986).

There also is a water-soluble version of this reagent available. Sulfo-BSOCOES [*bis*[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone] is built on the same chemical structure as BSOCOES, but contains the negatively charged sulfonate groups on both of its succinimide rings. The presence of the sulfonates provides enough charge and hydrophilicity to lend water solubility to the entire reagent. Thus, it may be added directly to aqueous reactions at concentrations of up to 10 mM. Prior dissolution in organic solvent, however, may provide solubility at greater concentrations in aqueous solutions.

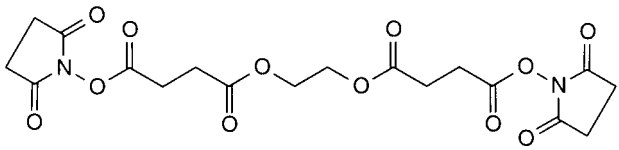
Additional applications of BSOCOES and sulfo-BSOCOES include investigations of the cellular and subcellular distribution of the type II vasopressin receptor (Fenton *et al.*, 2007), TNF-alpha (Grinberg *et al.*, 2005), and studying mechanisms in the control of plasmid replication (Das *et al.*, 2005).

1.5. EGS and Sulfo-EGS

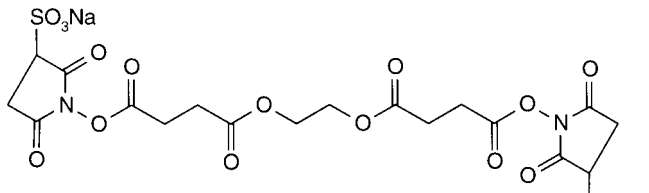
Ethylene glycol*bis*(succinimidylsuccinate) (EGS) is a homobifunctional crosslinking agent that contains NHS ester groups on both ends (Thermo Fisher). Its central bridge is constructed from an ethylene glycol group esterified on either side with succinic acid, the terminal carboxylates of which are activated by forming NHS esters. The two NHS esters are amine reactive, forming stable amide bonds between crosslinked molecules within a pH range of about

7–9. Avoid amine-containing buffers such as Tris or glycine, since they will cross-react with the NHS esters. Imidazole also should be avoided, because it has the effect of catalyzing the hydrolysis of the NHS ester groups. The internal structure of EGS provides two cleavable ester sites that may be broken at pH 8.5 by incubation with 1 M hydroxylamine for 3–6 hours at 37°C (Abdella *et al.*, 1979) (Figure 4.9). Thus, conjugates produced from the EGS crosslinking of the specific interaction of proteins or other molecules subsequently may be cleaved with hydroxylamine for analysis.

EGS is water-insoluble and must be dissolved in an organic solvent prior to its addition to an aqueous reaction. Prepare a concentrated solution of EGS in DMF or DMSO and add an aliquot of the stock solution to the reaction. Do not exceed a concentration of more than about 10 percent organic solvent in the aqueous reaction buffer or precipitation of buffer salts or protein may occur.



EGS
Ethylene glycolbis(succinimidylsuccinate)
MW 456.37
16.1 Å



Sulfo-EGS
Ethylene glycolbis(sulfosuccinimidylsuccinate)
MW 660.47
16.1 Å

Reported applications of EGS include crosslinking studies of cytochrome P-450 (Baskin and Yang, 1980a), conjugation of TNF with lymphotoxin (Browning and Ribolini, 1989), the conversion of a gonadotropin-releasing hormone antagonist to an agonist (Conn *et al.*, 1982a), preparation of a conjugate of gonadotropin-releasing hormone with an agonist (Conn *et al.*, 1982b), covalent crosslinking of vasoactive peptide to its lymphoblast receptors (Wood and O'Dorisio, 1985), and study of bombesin receptors in Swiss 3T3 cells (Millar and Rozengur, 1990).

A water-soluble analog of EGS also is available commercially (Thermo Fisher). Sulfo-EGS, or ethylene glycol*bis*(sulfosuccinimidylsuccinate), contains negatively charged sulfonate groups on its NHS rings. The resultant charge and hydrophilicity of this modification provides water solubility to the entire compound so that prior dissolution in organic solvent is not necessary.

EGS and sulfo-EGS also have been used to study the surface loop motion in FepA (Scott *et al.*, 2002), to characterize the high affinity copper transporter in Saccharomyces cerevisiae (Peña *et al.*, 2000), and in the study of protein interactions and large protein complexes (Petrotchenko *et al.*, 2005{2005 a or b}).

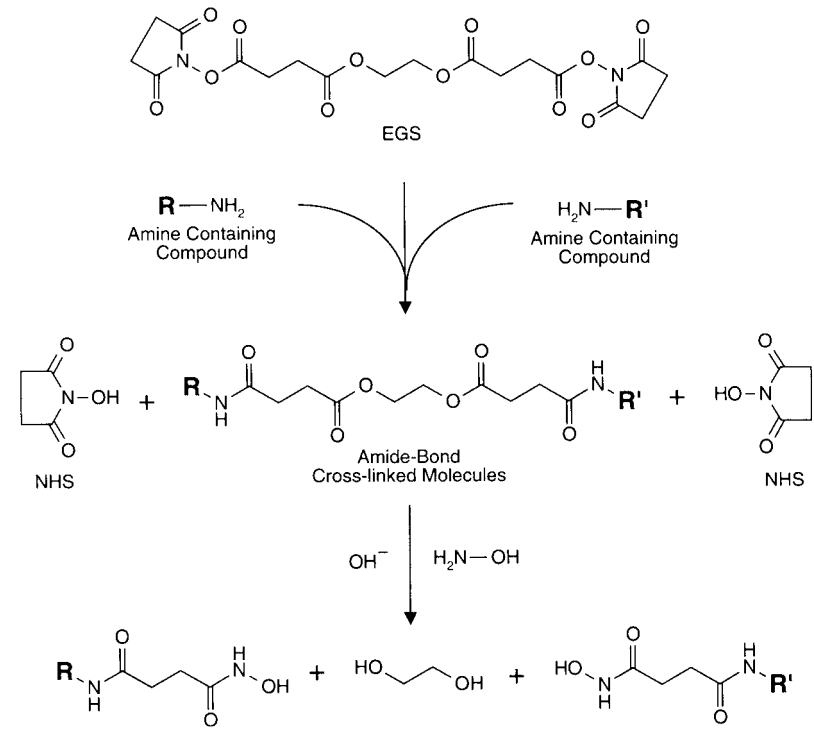
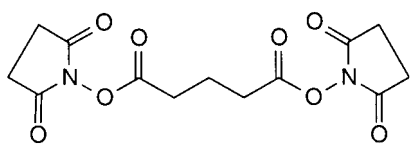


Figure 4.9 EGS reacts with amine-containing molecules to form amide linked conjugates. The ester groups within its cross-bridge are cleavable under alkaline conditions using hydroxylamine.

1.6. DSG

Disuccinimidyl glutarate (DSG) is a water-insoluble, homobifunctional crosslinker containing amine-reactive NHS esters at both ends (Thermo Fisher). The active esters react with amino groups in protein molecules in the pH range of 7–9 to form amide linkages. DSG is a non-cleavablereagent, forming stable 5-carbon bridges between amine-containing molecules (Figure 4.10).

DSG should be dissolved in an organic solvent prior to addition to an aqueous reaction medium. Suitable solvents include DMF and DMSO. To initiate a reaction, add an aliquot of the organic solution to the buffered medium containing the molecules to be crosslinked. Reaction buffers should not contain any competing amine compounds such as Tris or glycine,



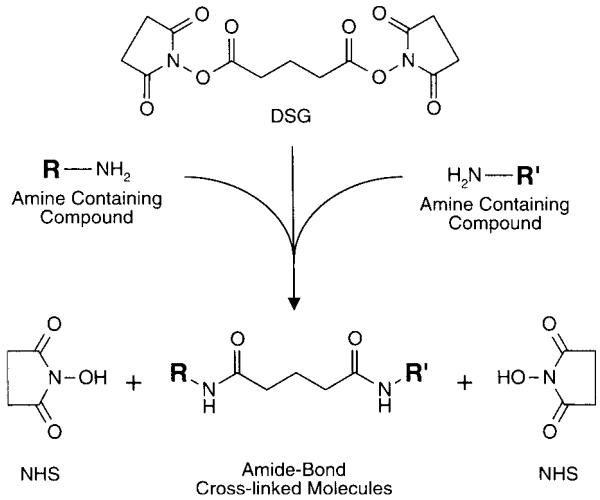


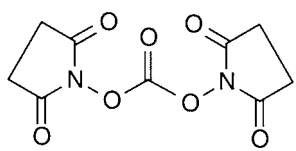
Figure 4.10 DSG is a non-cleavable crosslinker that can react with two amine-containing molecules to form amide bonds.

as these will cross-react with the active esters. Avoid imidazole-containing buffers, also, since it catalyzes the hydrolysis of NHS esters.

The reported applications of DSG include receptor–ligand studies by covalent crosslinking of their complexes (Waugh *et al.*, 1989), the capture of protein interactions on protein array surfaces by DSG crosslinking (MacBeath, 2007), studying TorT, a member of a periplasmic-binding protein family (Baraquet *et al.*, 2006), and in the investigation of left-helical conformation of l-DNA for analyzing biomarkers (Hauser *et al.*, 2006).

1.7. DSC

N,*N*′-Disuccinimidyl carbonate (DSC) is the smallest homobifunctional NHS ester crosslinking reagent available (Aldrich). It is, in essence, merely a carbonyl group-containing two NHS esters. The compound is highly reactive toward nucleophiles. In aqueous solutions, DSC rapidly will hydrolyze to form two molecules of NHS with release of CO_2. In nonaqueous environments, it can react with two amine groups to form a substituted urea derivative with loss of two molecules of NHS. The reagent also can be used in anhydrous organic solvents to activate a hydroxyl group to an amine-reactive succinimidyl carbonate derivative. This procedure is commonly used



N,N'-Disuccinimidyl Carbonate (DSC) MW 256.17

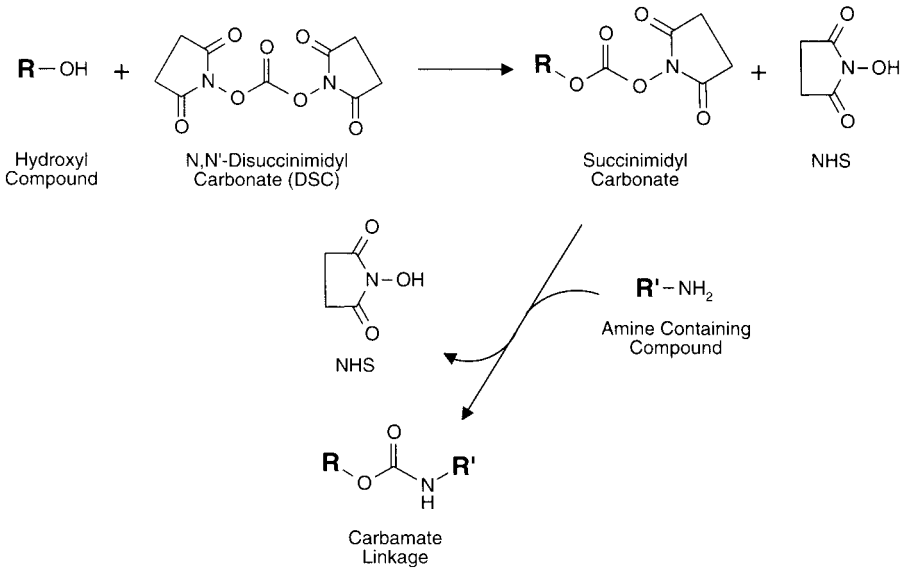


Figure 4.11 DSC can react with hydroxyl groups to create a succinimidyl carbonate intermediate that is highly reactive toward nucleophiles. In the presence of an amine-containing molecule, the active species can form stable carbamate linkages.

to activate poly(ethylene glycol) for conjugation with proteins and other molecules (Chapter 25, Section 1.2). In this sense, DSC-activated hydroxylic compounds can be used to conjugate with an amine-containing molecule to form a stable derivative (Figure 4.11). The linkage created from this reaction is a urethane derivative or a carbamate bond, displaying excellent stability.

Activation of hydroxyl groups with DSC can be done in acetone or dioxane by reacting for 4–6 hours at room temperature. Subsequent conjugation with amine-containing molecules is done in organic or aqueous solutions. For buffered reactions, the optimal conditions include a pH range of 7–9 using common buffer salts (avoid amine-containing components, such as Tris). React for at least 4 hours at room temperature or up to overnight at 4°C.

DSC also is used to activate hydroxylic particles for coupling to amine-containing ligands (Miron and Wilchek, 1993). For methods involving particle conjugation using this homobifunctional compound, see Chapter 14.

2. Homobifunctional Imidoesters

Crosslinking compounds-containing imidoesters at both ends are among the oldest homobifunctional reagents used for protein conjugation (Hartman and Wold, 1966). The imidoester (or imidate) functionality is one of the most specific acylating groups available for the modification of primary amines, with minimal cross-reactivity toward other nucleophilic groups in proteins. The α-amines and ε-amines of proteins may be targeted and crosslinked by reacting with homobifunctional imidoesters at a pH of 7–10 (optimal pH 8–9). The product of this reaction, an imidoamide (or amidine) (Figure 4.12), is protonated and thus carries a positive

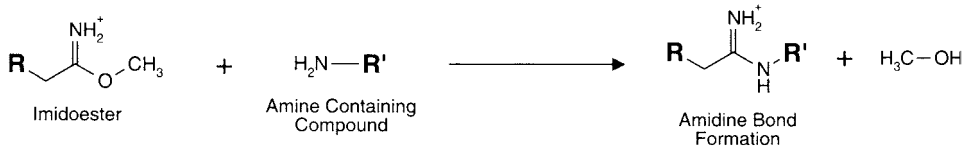


Figure 4.12 Imidoesters react with amine groups to form amidine bonds, which are positively charged at physiological pH.

charge at physiological pH (Kiehm and Ji, 1977; Liu *et al.*, 1977; Ji, 1979; Wilbur, 1992). The result is no alteration of the charge characteristics of the crosslinked proteins, since the amines being modified were themselves protonated and originally contributed to the overall positive charge of the molecule. Imidoesters therefore can preserve the microenvironment within the vicinity of the crosslink bridge, possibly retaining native structure and activity better than reagents that modulate the net charge of a protein.

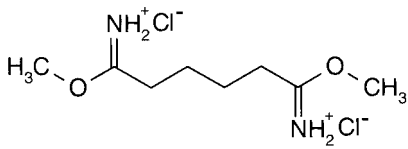
The amidine bond is quite stable at acid pH; however, it is susceptible to hydrolysis and cleavage at alkaline pH. Derivatized proteins may be assayed by amino acid analysis after acid hydrolysis without loss of imidate modifications.

Imidoester crosslinkers are highly water-soluble, but undergo continuous degradation due to hydrolysis. The half-life of the imidate functionality is typically less than 30 minutes, especially in the alkaline conditions of the reaction medium (Hunter and Ludwig, 1962; Browne and Kent, 1975). Concentrated stock solutions may be prepared before addition of a small amount to a conjugation reaction, but they should be dissolved rapidly and used immediately.

The following list of homobifunctional imidoesters represent compounds that are commonly used for protein crosslinking and are currently available from commercial sources.

2.1. DMA

Dimethyl adipimidate (DMA) is a short-chain, homobifunctional crosslinking agent-containing imidoesters at both ends (Thermo Fisher). After reaction with amine groups on target molecules, the compound creates a non-cleavable, 6-atom bridge with terminal amidine bonds (Figure 4.13). DMA is water-soluble and may be added directly to a crosslinking reaction or pre-dissolved at higher concentration before addition of an aliquot to the reaction medium. Stock solutions should be used immediately to prevent breakdown by hydrolysis. Reaction buffers having a pH of 8–9 are optimal. Avoid buffers-containing primary amines (glycine and



DMA
Dimethyl adipimidate dihydrochloride
MW 245.15
8.6 Å

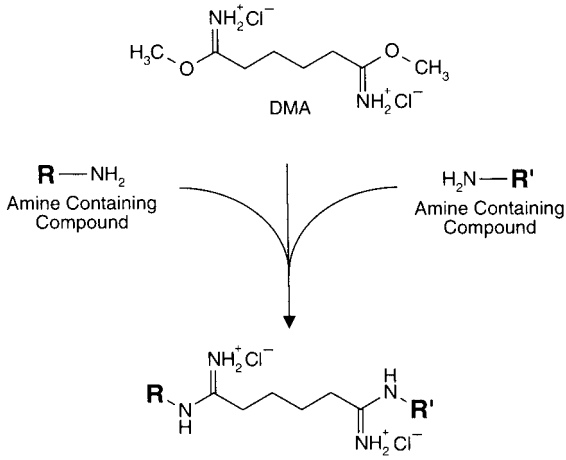


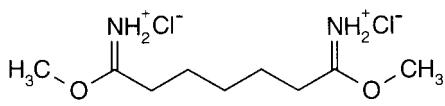
Figure 4.13 DMA can crosslink two amine-containing molecules to form charged amidine linkages.

Tris), since these will cross-react with the imidoester groups. Borate or bicarbonate buffers adjusted to the optimum pH range work well. The addition of (or the exclusive use of) 0.1–0.2 M triethanolamine is often done to help catalyze the coupling reaction.

Reported applications of DMA include the crosslinking of bovine pancreatic ribonuclease A (Hartman and Wold, 1967), treatment of erythrocyte membranes to reduce the effects of sickle cell anemia (Waterman *et al.*, 1975), conjugation and analysis of the outer membrane proteins of *Neisseria gonorrhoeae* (Newhall *et al.*, 1980), protein structural studies of bovine α-crystalline (Siezen *et al.*, 1980), crosslinking of hemoglobin S (Pennathur-Das *et al.*, 1982), and forming *S*-carbomethoxyvaleramidine during hydrolysis of DMA (Mentzer *et al.*, 1982). The compound also has been used to study uranyl–antibody interactions by atomic force microscopy (Odorico *et al.*, 2007), to produce antibody–drug conjugates for the treatment of non-Hodgkin lymphoma (Polson *et al.*, 2007), and to investigate the subcellular distribution of the type II vasopressin receptor in kidney (Fenton *et al.*, 2007).

2.2. DMP

Dimethyl pimelimidate (DMP) is a homobifunctional crosslinking agent that has imidoester groups on either end (Thermo Fisher). The imidoesters are amine reactive to give stable amidine linkages with target molecules. The 7-atom bridge created by DMP crosslinks is non-cleavable and



DMP
Dimethyl pimelimidate dihydrochloride
MW 259.18
9.2 Å

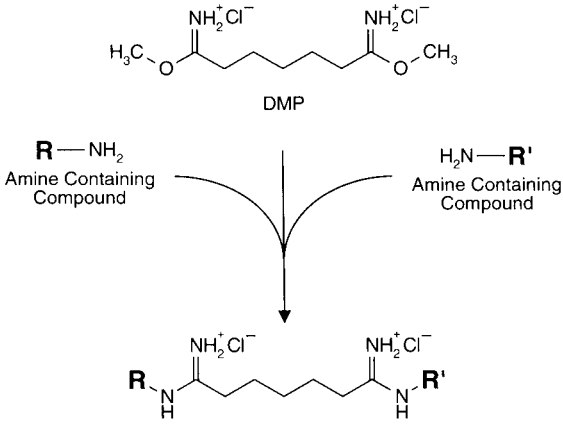


Figure 4.14 DMP reacts with amine-containing compounds to form amidine bonds.

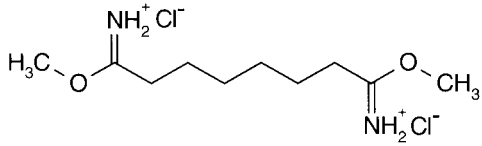
positively charged at physiological pH due to the protonated amidine bonds (Figure 4.14). The reagent is water-soluble and may be reacted with proteins or other amine-containing macromolecules at a pH of 8–9 in aqueous media. Use buffers that contain no amine groups that may cross-react with the imidoesters. Avoid glycine or Tris buffers.

In protein crosslinking studies, DMP has been used to examine the subunit structure of muscle pyruvate kinase (Davies and Kaplan, 1972), for the crosslinking of lactose synthetase (Brew *et al.*, 1975), and to conjugate a fluorescent derivative of α-lactalbumin to glactosyltransferase (O'Keefe *et al.*, 1980). The reagent also has found use in the immobilization of antibody molecules to insoluble supports containing bound protein A (Schneider *et al.*, 1982). The antibody molecules are first allowed to interact with the coupled protein A, orienting them with their antigen-binding sites facing away from the matrix. DMP is then added to covalently anchor the antibodies to the protein A, forming a permanent immunoaffinity matrix.

DMP also has been used to study the interaction between the endoplasmic reticulum and microtubules (Ogawa-Goto *et al.*, 2007), the conformational changes in the outer arm dynein of Chlamydomonas in response to calcium (Sakato *et al.*, 2007), and the secretion of the adipocyte-specific secretory protein adiponectin (Wang *et al.*, 2007).

2.3. DMS

Dimethyl suberimidate, DMS, is a homobifunctional crosslinking agent-containing amine-reactive imidoester groups on both ends. The compound is reactive toward the ε-amine groups



DMS
Dimethyl suberimidate dihydrochloride
MW 273.2
11 Å

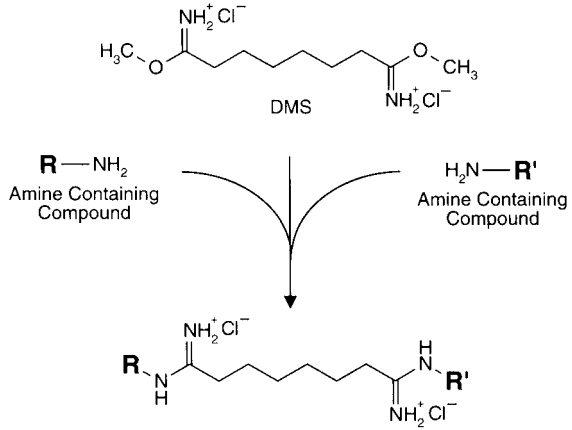


Figure 4.15 The reaction of DMS with amine-containing molecules yields amidine linkages.

of lysine residues and N-terminal α-amines in the pH range of 7–10 (pH 8–9 is optimal). The resulting amidine linkages are positively charged at physiological pH, thus maintaining the positive charge contribution of the original amine. DMS creates 8-atom bridges between conjugated molecules that are not cleavable (Figure 4.15).

DMS reportedly has been used as a tissue fixative for light and electron microscopy (Hassell and Hand, 1974), for the study of the subunit structure of oligomeric proteins (Davies and Stark, 1970), in the investigation of ATPase activity (Adolfson and Moudrianokis, 1976), crosslinking ribonuclease A (Wang *et al.*, 1976), binding studies of nerve growth factor to its receptor (Pulliam *et al.*, 1975), in the study of red cell shape (Mentzer and Lubin, 1979), crosslinking of glycogen phosphorylase b (Hajdu *et al.*, 1979), crosslinking of apo low density lipoproteins (Ikai and Yanagita, 1980), studying the mechanism of binding of multivalent immune complexes to Fc receptors (Dower *et al.*, 1981), investigating the quaternary structure of the pyruvate dehydrogenase multi-enzyme complex of *Bacillus stearothermophilus* (Packman and Perham, 1982), crosslinking studies of the protein topography of rat liver microsomes (Baskin and Yang, 1982), affinity crosslinking studies of the protein topography of rat liver microsomes (Pfeuffer *et al.*, 1985), and the quantitative chemical crosslinking of CAD protein (Lee *et al.*, 1985).

DMS also has been used to study the interaction between the Helicobacter pylori accessory proteins HypA and UreE (Benoit *et al.*, 2007) as well as to identify the interaction between the Ca^{2+}-binding protein S100A11 and the Ca^{2+}- and phospholipid-binding protein annexin A6 (Chang *et al.*, 2007).

2.4. DTBP

Dimethyl 3,3′-dithiobispropionimidate (DTBP) is a homobifunctional, reversible crosslinking agent-containing imidoester groups on both ends (Thermo Fisher). The compound, commonly called Wang and Richards' reagent, is water-soluble, and reacts with amines in the pH range of 7–10 (optimum 8–9) to produce amidine linkages (Wang and Richards, 1974). Conjugated

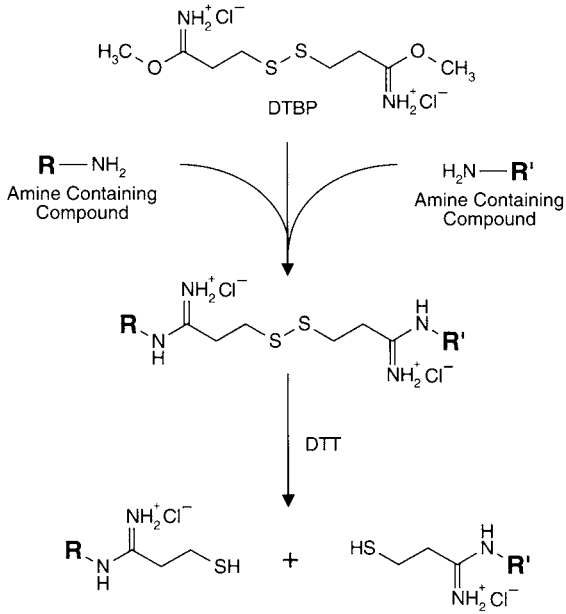
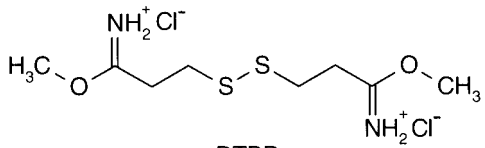


Figure 4.16 DTBP reacts with amine-containing molecules to form charged amidine bonds. The internal disulfide group can be cleaved with DTT to release the conjugate.

molecules subsequently may be cleaved by reduction of the internal disulfide group of the 8-atom bridge (Figure 4.16). Crosslinked molecules may be analyzed by one- or two-dimensional electrophoresis, making use of the easy reversibility of the disulfide bond.



DTBP
Dimethyl-3,3'-dithiobispropionimidate dihydrochloride
MW 309.28
11.9 Å

Reported applications include studying protein–protein interactions with paramyxoviruses (Markwell and Fox, 1980), inhibiting adenylate cyclase activity (Young, 1979), studying red cell shape (Mentzer and Lubin, 1979), crosslinking phytochrome to its putative receptor (Yu and Schweinberger, 1979), investigating the subunit structure of Na, K-ATPase (dePont, 1979), Newcastle disease virus proteins (Nagai *et al.*, 1978), thylakoid membrane proteins (Novak-Hofer and Siegenthaler, 1978), glutamate dehydrogenase–amino transferase complexes (Fahien *et al.*, 1978), vesicular stomatitis virus proteins (Mudd and Swanson, 1978), heamagglutinin of influenza virus (Wiley *et al.*, 1977), pig heart lactate dehydrogenase crystals (Bayne and Ottesen, 1977), beef heart mitochondrial coupling factor 1 (Baird and Hammes, 1977), chloroplast coupling factor 1 (Baird and Hammes, 1976), rabbit muscle skeletal sarcoplasmic reticulum

protein (Louis *et al.*, 1977), human hemoglobin and erythrocyte membrane proteins (Wang and Richards, 1974, 1975; Miyakawa *et al.*, 1978), subunit interface of the E. coli ribosome (Cover *et al.*, 1981), rat liver 60S ribosomal subunits (Uchiumi *et al.*, 1980), proteins in avian sarcoma and leukemia viruses (Pepinsky *et al.*, 1980), outer membrane proteins of Neisseria gonorrhoeae (Newhall *et al.*, 1980), monooxygenase enzymes (Baskin and Yang, 1980b), cytochrome P-450 and reduced NAD phosphate-cytochrome P-450 reductase (Baskin and Yang, 1980b), crosslinking initiation factor IF2 to proteins in 70S ribosomes of *E. coli* (Heinmark *et al.*, 1976), studying sheep red blood cell membranes (Brandon, 1980), conjugation of F-actin to skeletal muscle myosin subfragment-1 (Labbe *et al.*, 1982), studying decreased staining of proteins after electrophoresis (Leffak, 1983), and identifying molecular association of IA antigens after T- and B-cell interaction (Shivdasani and Thomas, 1988).

DTBP also has been used to investigate the dimerization and actin bundling properties of villin (George *et al.*, 2007), the interaction of the Mre11 complex with RPA (Olson *et al.*, 2007), the study of gamma-secretase complex assembly (Spasic *et al.*, 2007), and the multi-protein assembly of Kv4.2, KChIP3 and DPP10 (Jerng *et al.*, 2005).

3. Homobifunctional Sulfhydryl-Reactive Crosslinkers

Crosslinking agents that contain homobifunctional sulfhydryl-reactive groups at either end fall into two general categories: those that form permanent bonds with available sulfhydryls and those that create reversible linkages. Reactive groups yielding permanent links with sulfhydryls usually form thioether bonds that are quite stable. Those that result in disulfide bonds can be cleaved with the use of a disulfide reducing agent like DTT (Chapter 1, Section 4.1). Mercurial-based coupling groups also can be reversed with reducing agents.

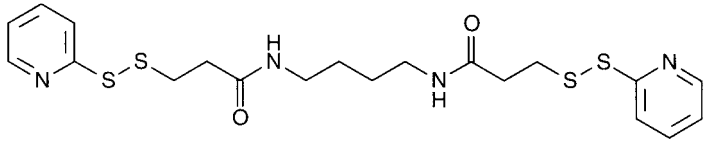
Many varieties of homobifunctional, sulfhydryl-reactive crosslinkers have been synthesized and described in the literature. Some have been based on *bis*-mercurial salts (Edelhoch *et al.*, 1953; Edsall *et al.*, 1954; Kay and Edsall, 1956; Singer *et al.*, 1960; Mandy *et al.*, 1961). Such mercurial-reactive groups also have been used in reversible covalent chromatography applications to purify thiol-containing proteins (Cuatrecasas, 1970, 1972; Ruiz-Carrillo and Allfrey, 1973). Other homobifunctional sulfhydryl-reactive reagents have been based on forming a mixed disulfide active group with TNB (5-thio-2-nitrobenzoic acid). Reaction of the TNB active group with a sulfhydryl-containing macromolecule results in a reversible disulfide linkage (Gaffney *et al.*, 1983; Willingham and Gaffney, 1983). Active groups consisting of bis-thiosulfonates also have been used to create—SH-reactive crosslinkers (Bloxham and Sharma, 1979; Bloxham and Cooper, 1982). The thiosulfonate groups react with available sulfhydryls to form disulfide linkages, in this case with loss of the sulfonate groups. All of these disulfide crosslinks are cleavable with disulfide reducing agents.

A number of bis-alkyl halide-reactive groups have been used to create homobifunctional sulfhydryl-reactive crosslinkers (Ozawa, 1967; Husain and Lowe, 1968; Wilchek and Givol, 1977). These react with sulfhydryls to create stable, nonreversible thioether bonds. Similar thioether bond formation has been realized using various bis-maleimide derivatives (Moore and Ward, 1956; Kovacic and Hem, 1959; Tawney *et al.*, 1961; Fasold *et al.*, 1963; Simon and Konigsberg, 1966; Zahn and Lumper, 1968; Freedberg and Hardman, 1971; Chang and Flaks, 1972; Wells *et al.*, 1980; Heilmann and Holzner, 1981; Sato and Nakao, 1981; Moroney *et al.*, 1982; Partis *et al.*, 1983; Chantler and Bower, 1988; Srinivasachar and Neville, 1989). Sulfhydryls add to the double bond of the maleimide to create a thioether linkage.

The differences within these families of reagents generally relate to the length of the spacer or bridging portion of the molecule. Occasionally, the bridging portion itself is designed to be cleavable by one of a number of methods (Chapter 8). The great majority of homobifunctional, sulfhydryl-reactive crosslinkers mentioned in the literature are not readily available from commercial sources and would have to be synthesized to make use of them. The ones listed in this section are obtainable from Thermo Fisher.

3.1. DPDPB

DPDPB, or 1,4-di-[3′-(2′-pyridyldithio)propionamido]butane, is a homobifunctional crosslinking agent that contains sulfhydryl-reactive dithiopyridyl groups on both ends. These coupling groups are identical to the sulfhydryl-reactive end of the popular heterobifunctional crosslinking agent, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Chapter 5, Section 1.1). Available thiols on proteins and other molecules can react with the pyridyl disulfide groups to form disulfide linkages with release of pyridine-2-thione (Figure 4.17). Conjugation of two macromolecules with DPDPB results in a 14-atom spacer of approximately 16 Å in length. Release of two molecules of pyridine-2-thione during the crosslinking reaction may be followed by their characteristic absorbance at 343 nm ($\varepsilon = 8.08 \pm 0.3 \times 10^3 M^{-1}cm^{-1}$) (Stuchbury *et al.*, 1975).



DPDPB
1,4-di-[3'-(2'-pyridyldithio)propionamido]butane
MW 482.7
19.9 Å

DPDPB is insoluble in aqueous solutions and should be initially dissolved in an organic solvent prior to addition of a small aliquot to a buffered reaction medium. Preparation of a stock solution in DMSO at a concentration of 25 mM DPDPB works well. The addition of an aliquot of this stock solution to the conjugation reaction should not result in more than about 10 percent organic solvent by volume in the buffered mixture or protein precipitation may occur.

DPDPB has two absorbance maxima, one peak at 237 nm ($\varepsilon = 1.2 \times 104 M^{-1}cm^{-1}$) and another at 287 nm ($\varepsilon = 8.8 \times 10^3 M^{-1}cm^{-1}$) (Traut *et al.*, 1989; Zecherle, 1990). Reduction of the pyridyldithio groups causes a shift in the absorbance characteristics of the molecule, such that the peak at 237 nm is shifted to 272 nm and the peak at 287 nm is shifted to 343 nm. This absorbance shift correlates to the release of the pyridine-2-thione groups.

DPDPB has been used to study the endocytosis of cadherin from intracellular junctions (Troyanovsky *et al.*, 2006), the subunit arrangement in the flagellar rotor assembly (Lowder *et al.*, 2005), and the disease-associated mutations in myelin proteolipid protein in the endoplasmic reticulum (ER) (Swanton *et al.*, 2005). DPDPB can be used to conjugate reduced antibody molecules to β-D-galactosidase using essentially the same protocol as that described by O'Sullivan *et al.* (1979).

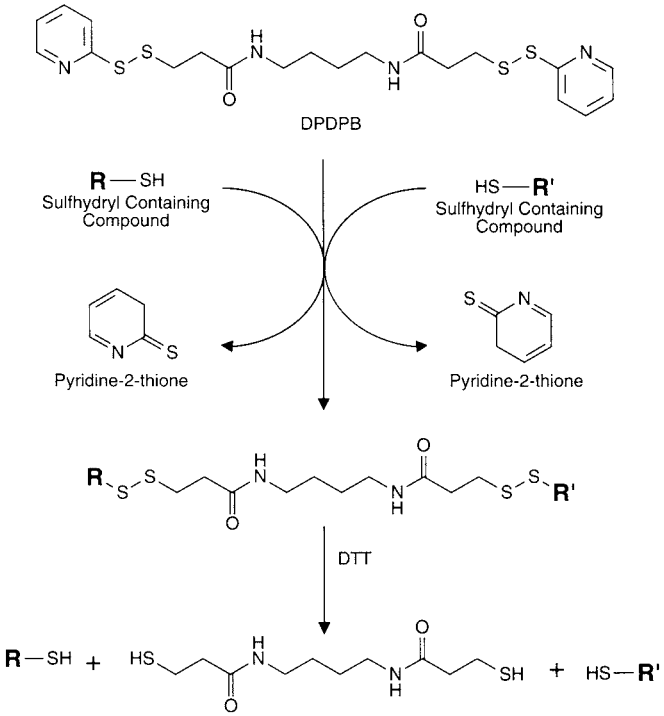
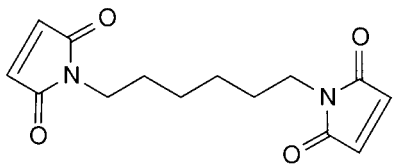


Figure 4.17 DPDPB is a sulfhydryl-reactive crosslinker that forms disulfide bonds with thiol-containing molecules. The conjugates may be disrupted using a disulfide reducing agent such as DTT.

3.2. BMH

Homobifunctional crosslinking compounds-containing maleimide groups on both ends have been used for quite some time (Moore and Ward, 1956; Kovacic and Hem, 1959; Tawney *et al.*, 1961; Fasold *et al.*, 1963; Simon and Konigsberg, 1966; Zahn and Lumper, 1968; Freedberg and Hardman, 1971; Chang and Flaks, 1972; Wells *et al.*, 1980; Heilmann and Holzner, 1981; Sato and Nakao, 1981; Moroney *et al.*, 1982; Partis *et al.*, 1983; Chantler and Bower, 1988; Srinivasachar and Neville, 1989).

Bis-maleimidohexane (BMH) is a homobifunctional reagent containing a non-cleavable, 6-atom spacer between terminal maleimides (Thermo Fisher). The maleimide groups can react



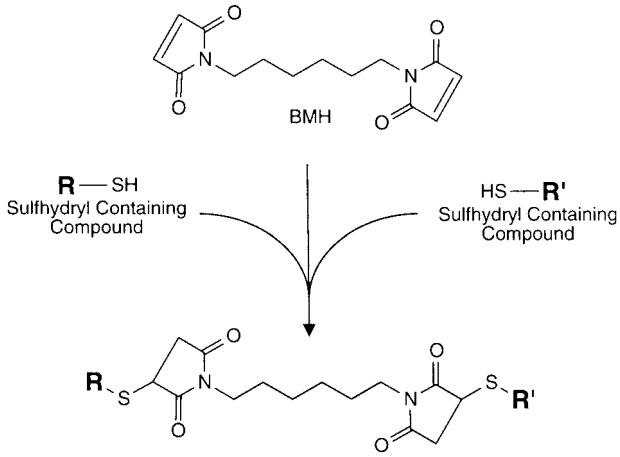


Figure 4.18 BMH contains two maleimide groups specific for crosslinking sulfhydryl-containing molecules. The thioether bonds that are formed are stable.

with sulfhydryls to form stable thioether linkages (Figure 4.18). Crosslinks formed with this reagent create a 16.1-Å bridge between conjugated macromolecules. The reaction takes place optimally from pH 6.5–7.5. Within this pH range, the reaction is very specific for sulfhydryls. At higher pH values, cross-reactivity with amino groups may occur (see Chapter 2, Section 2.2).

4. Difluorobenzene Derivatives

Difluorobenzene derivatives are small homobifunctional crosslinkers that react with amine groups. Conjugation using these compounds results in bridges of only about 3 Å in length, potentially providing information concerning very close interactions between macromolecules.

4.1. DFDNB

DFDNB is the acronym for an aryl halide-containing compound having the structural names, 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene (Thermo Fisher). The reagent

DFDNB
1,5-Difluoro-2,4-dinitrobenzene
MW 204.1
3 Å

contains two reactive fluorine atoms that can couple to amine-containing molecules, yielding stable arylamine bonds (Figure 4.19). However, the reactivity of aryl halides is not totally specific for amines. Thiol, imidazolyl, and phenolate groups of amino acid side chains also can react (Zahn and Meinhoffer, 1958). Conjugates formed with sulfhydryl groups, however, are reversible by cleaving with an excess of thiol (such as DTT) (Shaltiel, 1967). The compound is especially useful in crosslinking cellular membrane proteins, since it is able to penetrate the hydrophobic regions of the lipid bilayer.

Difluorobenzene reagents have been used for crosslinking phospholipids in human erythrocyte membranes (Berg *et al.*, 1965; Marfey and Tsai, 1975), conjugation of small peptides to the carrier protein albumin (Tager, 1976), studying the interaction of proteins in the myelin membrane (Golds and Braun, 1978), crosslinking of cytochrome oxidase subunits (Kornblatt and Lake, 1980), and studying the conformational effects of calcium on troponin C (Kareva *et al.*, 1986). DFDNB also has been used to investigate the conformational changes in Chlamydomonas outer arm dynein in response to calcium ions (Sakato *et al.*, 2007), to study the dimerization properties of villin (George *et al.*, 2007), and to characterize the intraflagellar transport complex B core (Lucker *et al.*, 2005).

4.2. DFDNPS

DFDNPS (4,4′-difluoro-3,3′-dinitrophenylsulfone) is a di-aryl halide reagent containing a central sulfone group (Figure 4.20). The aromatic fluorines are reactive with amines, sulfhydryls, phenolates, and imidazolyl groups of proteins (see previous section). The reaction with amines forms stable arylamine linkages. The reaction with sulfhydryl groups, however, is reversible by treatment with an excess of thiol. The central sulfone group provides cleavability through hydrolysis with base at pH 11–12 at 37°C (Wold, 1961, 1972; Zarling *et al.*, 1980).

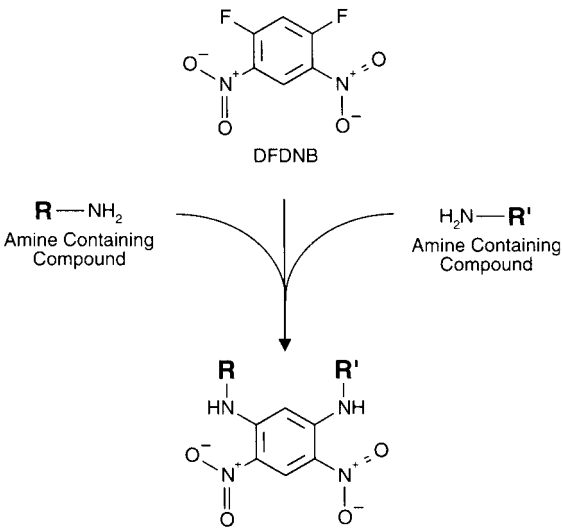
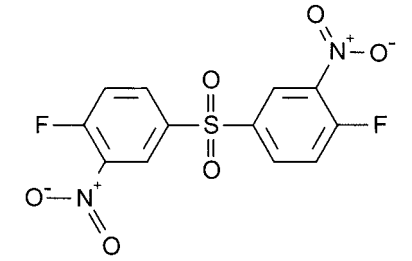


Figure 4.19 DFDNB is a small crosslinker able to form covalent bonds between amine-containing molecules. The aromatic fluorine atoms are readily displaced by nucleophiles.



5. Homobifunctional Photoreactive Crosslinkers

Although there are a number of photo-sensitive coupling chemistries that have been used in modification and conjugation reactions (Chapter 2, Section 7), it has been primarily aryl azides

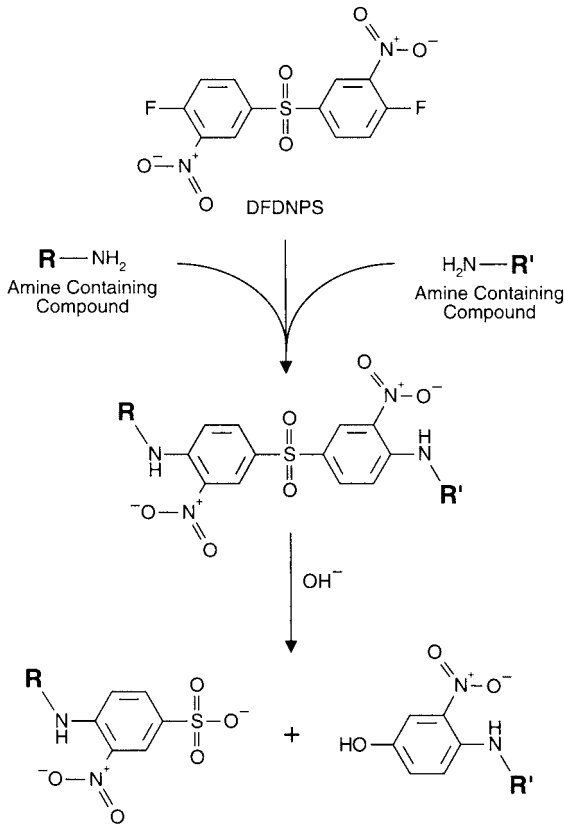


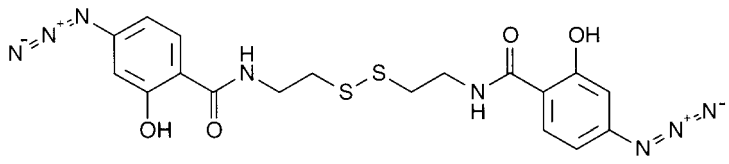
Figure 4.20 DFDNPS reacts with amine-containing molecules to form arylamine crosslinks. The central sulfone group in the cross-bridge can be cleaved under alkaline conditions.

that have found application in homobifunctional crosslinkers. The photolysis reaction requires exposure of the phenyl azide to a bright light source at a wavelength of 265–275 nm (Ji, 1979). If the aromatic ring contains a nitro group *meta* to the azide functionality, then photolysis can occur at higher wavelengths (300–460 nm). The photolysis process initially forms a highly reactive aryl nitrene, but these quickly undergo ring expansion to create a dehydroazepine. This active species principally reacts with nucleophiles, rather than inserting in C—H or N—H bonds or adding to double bonds. Thus, instead of nonselective coupling into nearly any part of a molecular structure, aryl azides ultimately react with primary amines more than any other functionality (Schnapp *et al.*, 1993).

Reported structures for homobifunctional aryl azides include a biphenyl derivative and a naphthalene derivative (Mikkelsen and Wallach, 1976), a biphenyl derivative containing a central, cleavable disulfide group (Guire, 1976), and a compound containing a central 1,3-diamino-2-propanol bridge between phenyl azide rings that are nitrated (Guire, 1976). The only commercially available homobifunctional photoreactive crosslinker is BASED.

5.1. BASED

Bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED) is a homobifunctional photoreactive crosslinking agent-containing phenyl azide groups at both ends (Thermo Fisher). Its central bridge contains a cleavable disulfide bond that may be broken after conjugation with the appropriate reducing agent (Chapter 1, Section 4.1). The aryl azides are salicylate derivatives that contain hydroxylic functions that activate the ring toward electrophilic reactions. Thus, the phenolic rings are modifiable with ^{125}I using traditional oxidative radioiodination reagents. Prior to the photoreactive conjugation step, the crosslinker may be iodinated with IODO-GEN or IODO-BEADS (Chapter 12, Sections 2 and 3). After two proteins are crosslinked, cleavage of the conjugate with DTT releases the link but maintains a radiolabel on each of the molecules (Figure 4.21).



BASED
Bis-[β-(4-azidosalicylamido)ethyl]disulfide
MW 474.54

6. Homobifunctional Aldehydes

Numerous *bis*-aldehyde reagents have been used for the conjugation of biomolecules. Nearly every small organic compound-containing two aldehyde groups has been at least tried in crosslinking reactions. The repertoire of available homobifunctional aldehydes ranges from the single-carbon formaldehyde (Section 6.1, this chapter; yes, it behaves as if it were bifunctional) through the 2-carbon atom glyoxal (Brooks and Klamerth, 1968), the 3-carbon malondialdehyde (Cater, 1963), the 4-carbon succinaldehyde (Cater, 1963), the popular 5-carbon

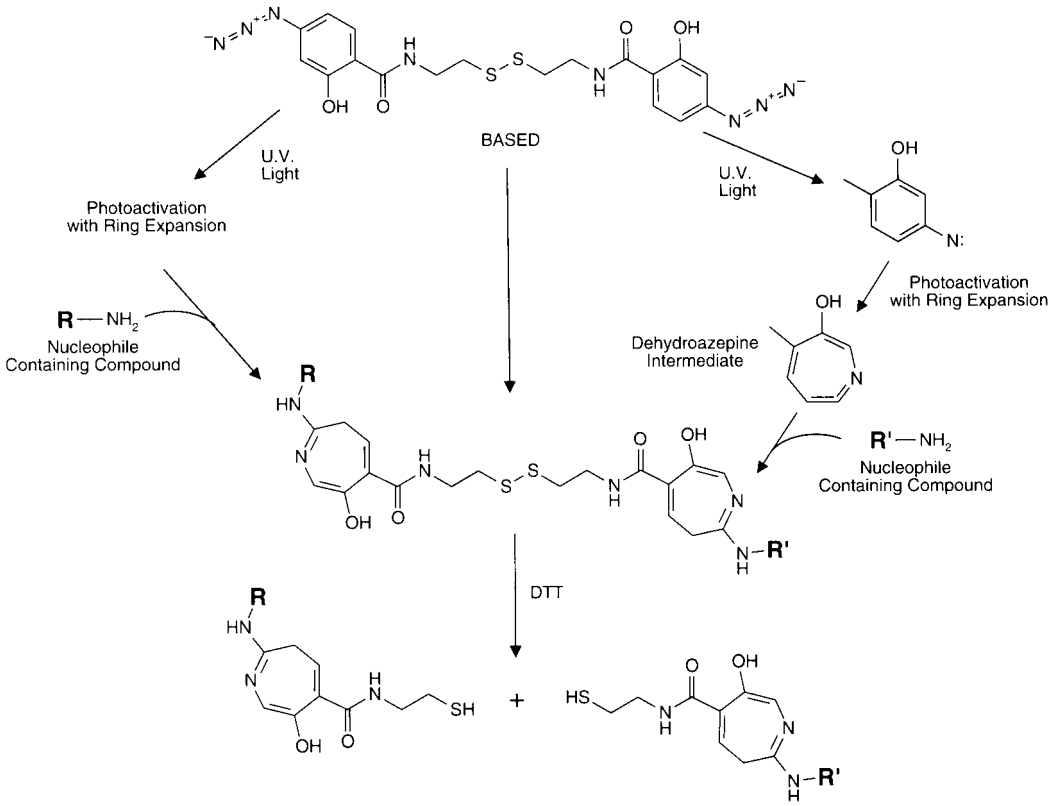


Figure 4.21 BASED can react with molecules after photoactivation to form crosslinks with nucleophilic groups, primarily amines. Exposure of its phenyl azide groups to UV light causes nitrene formation and ring expansion to the dehydroazepine intermediate. This group is highly reactive with amines. The cross-bridge of BASED is cleavable using a disulfide reducing agent.

glutaraldehyde (Section 6.2, this chapter), the 6-carbon adipaldehyde as well as its α-hydroxy derivative (Fein and Filachione, 1957; Seligsberger and Sadlier, 1957; Cater, 1963; Richard and Knowles, 1968; Hopwood, 1969), and several pyridoxal-polyphosphate derivatives that are internally cleavable with acid or base (Shimomura and Fukui, 1978; Benesch and Kwong, 1988).

By far, the two most popular *bis*-aldehyde reagents are formaldehyde and glutaraldehyde.

6.1. Formaldehyde

Formaldehyde is the smallest crosslinking reagent available that does not create a zero-length bridge between two molecules (Chapter 3). Although technically not a homo-bifunctional reagent, it undergoes crosslinking reactions as though it possessed two functional groups. In concentrated aqueous solutions, it can form the low-molecular-weight polymers typically observed

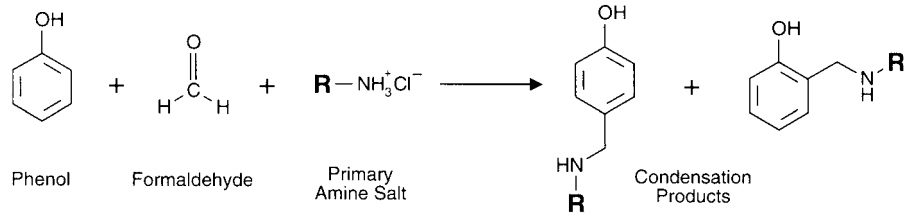
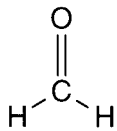


Figure 4.22 The Mannich reaction occurs between an active-hydrogen-containing compound (phenol) and an amine-containing molecule in the presence of an aldehyde (formaldehyde). The condensation reaction forms stable crosslinks.

in formalin preparations. In dilute solutions, it exists mainly in its monomeric state. Older solutions of formaldehyde may contain precipitated polymer that often can be resolubilized by heating. Commercial preparations of formaldehyde may be obtained as a 37 percent solution stabilized against polymerization by the addition of methanol.

Conjugation reactions using formaldehyde may proceed by one of two routes: the Mannich reaction or via an immonium cation intermediate. The Mannich reaction consists of the condensation of formaldehyde (or sometimes another aldehyde) with ammonia (in the form of its salt) and another compound containing an active hydrogen. For a review of this reaction mechanism, see Adams *et al.* (1942). Instead of ammonia, however, this reaction can be done with primary or secondary amines, or even with amides. An example of this is illustrated in Figure 4.22 by the condensation of phenol, formaldehyde, and a primary amine salt (the active hydrogens are shown underlined). Figure 4.23 shows some active-hydrogen-containing functional groups that can participate in the Mannich reaction.



Formaldehyde
MW 30

The Mannich reaction can be used for the immobilization of certain drugs, steroidal compounds, dyes, or other organic molecules that do not possess the typical nucleophilic groups able to participate in traditional coupling reactions (Hermanson *et al.*, 1992). It also can be used to conjugate hapten molecules to carrier proteins when the hapten contains no convenient nucleophile for conjugation (Chapter 19, Section 6.2). In this case, the carrier protein contains the primary amines and the hapten contains at least one sufficiently active hydrogen to participate in the condensation reaction.

To obtain acceptable yields, the Mannich reaction must be done at elevated temperatures. Incubation at 37–57°C for at least 2–24 hours usually is required to complete the reaction. Addition of formaldehyde is done by adding an aliquot of a 37 percent solution to the reaction to obtain about a 10- to 100-fold molar excess over t he amount of active-hydrogen-containing

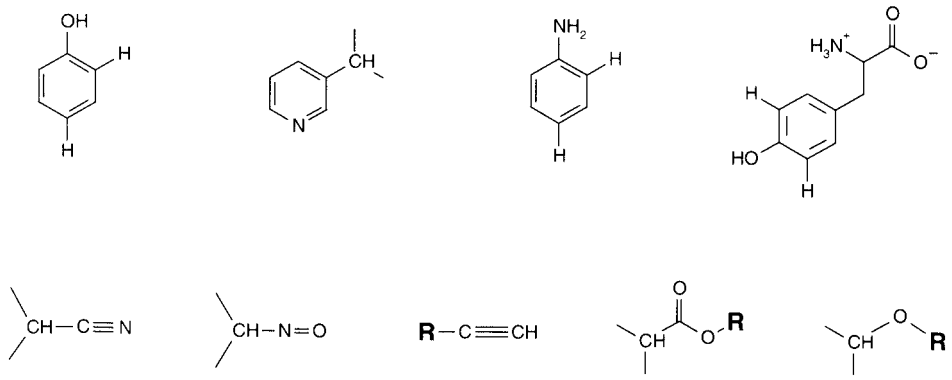


Figure 4.23 Examples of active-hydrogen-containing compounds that can participate in the Mannich reaction. The points of reactivity are shown by the hydrogen atoms.

compound to be conjugated. Thermo Fisher has designed a kit for the conjugation of haptens to carrier proteins using the Mannich reaction mechanism.

A secondary reaction pathway also is possible in formaldehyde-facilitated conjugations. Formaldehyde may react with a primary amine to form a quaternary ammonium salt. This intermediate spontaneously reacts to create a highly active immonium cation with loss of one molecule of water (Blass *et al.*, 1965; Ji, 1983). The immonium cation is reactive toward nucleophiles in proteins and other molecules, including amines, sulfhydryls, phenolic groups, and imidazole nitrogens. The reaction yields methylene bridges between two nucleophiles, binding macromolecules with a one-carbon linker (Figure 4.24).

It is obvious that the Mannich reaction pathway and the immonium ion mechanism may occur simultaneously, especially at conditions of room temperature or greater. Formaldehyde-facilitated crosslinking reactions between molecules that both contain nucleophiles probably occur primarily by the immonium ion pathway, since the Mannich reaction proceeds at a slower rate. In addition, the Mannich reaction will cause nondescript polymerization between molecules that possess both active hydrogens and amine groups. It is best to utilize the Mannich reaction only when one of the molecules contains no nucleophilic groups but at least one active hydrogen, and the other molecule contains a primary or secondary amine.

Formaldehyde also can be used to study protein interactions in cells or tissue sections by crosslinking and capturing protein complexes. Chapter 28, Section 1.3 describes this method and contains a protocol for use.

6.2. Glutaraldehyde

Glutaraldehyde is the most popular *bis*-aldehyde homobifunctional crosslinker in use today. However, a glance at glutaraldehyde's structure is not indicative of the complexity of its possible reaction mechanisms. Reactions with proteins and other amine-containing molecules would be expected to proceed through the formation of Schiff bases. Subsequent reduction with sodium cyanoborohydride or another suitable reductant would yield stable secondary amine

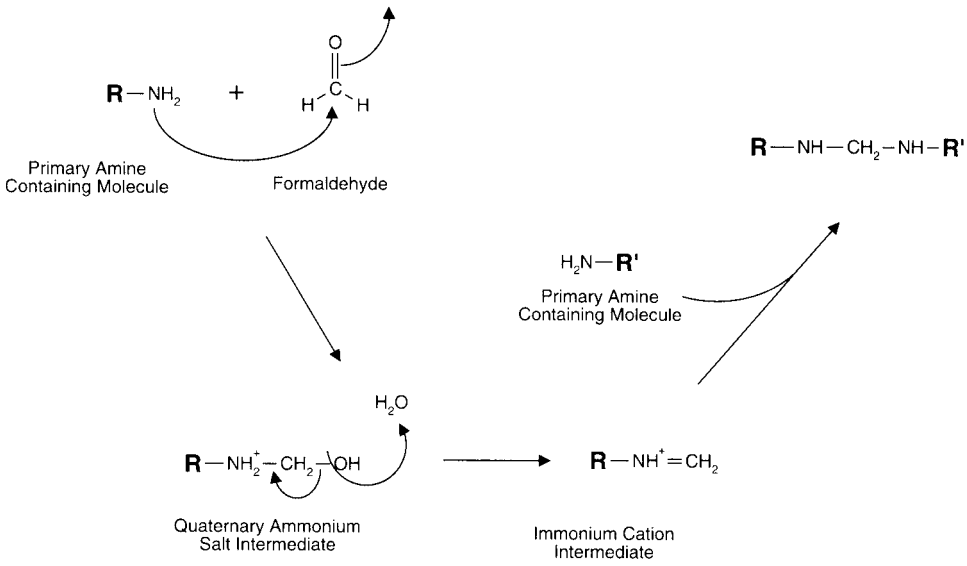
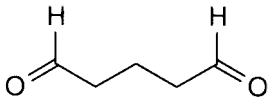


Figure 4.24 Two amine-containing molecules can be crosslinked by formaldehyde through formation of a quaternary ammonium salt with subsequent dehydration to an immonium cation intermediate. This active species then can react with a second amine compound to form stable secondary amine bonds.

linkages (Chapter 2, Section 5.3 and Chapter 3, Section 4). This reaction sequence certainly is possible, but other crosslinking reactions also occur.



Glutaraldehyde
MW 100.11

Glutaraldehyde in aqueous solutions can form polymers-containing points of unsaturation (Hardy *et al.*, 1969, 1976; Monsan *et al.*, 1975) (Figure 4.25). Such α,β-unsaturated glutaraldehyde polymers are highly reactive toward nucleophiles, especially primary amines. Reaction with a protein results in alkylation of available amines, forming stable secondary amine linkages. These glutaraldehyde-modified proteins still may react with other amine-containing molecules either through the Schiff base pathway or through addition at other points of unsaturation (Figure 4.26). The proposed reaction mechanism of conjugation using these polymer conjugates explains the stability of proteins crosslinked by glutaraldehyde that have not been reduced. Schiff base formation alone would not yield stable crosslinked products without reduction.

Crosslinking using glutaraldehyde polymers is difficult to reproduce. Since the glutaraldehyde polymer size and structure is unknown, the exact nature of the conjugates formed by this method is indeterminable, as well. The age of a glutaraldehyde solution is another variable, because the older the solution the more polymer will be formed. Fresh glutaraldehyde often will not yield the same results as aged solutions.

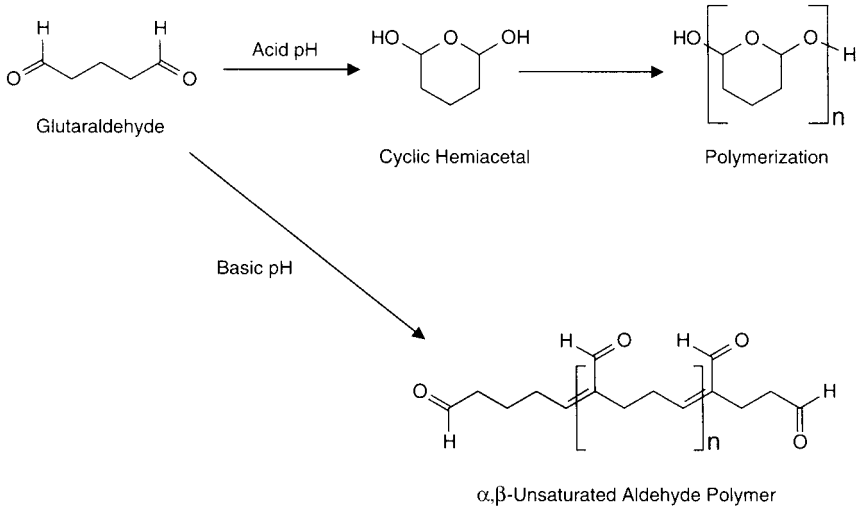


Figure 4.25 Glutaraldehyde in aqueous solution may polymerize at either acid or basic pH.

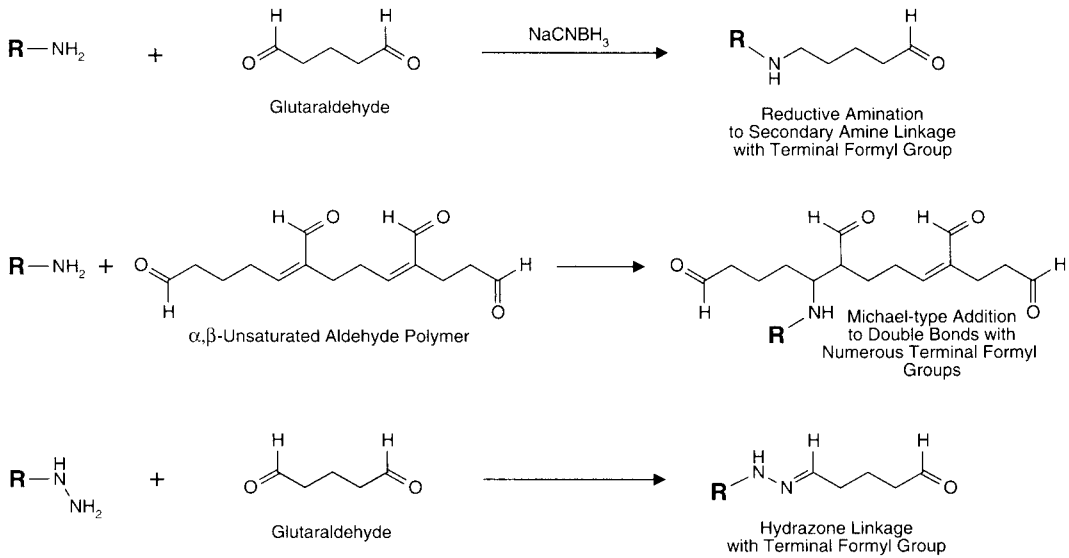


Figure 4.26 Glutaraldehyde may react by several routes to form covalent crosslinks with amine-containing molecules.

A third method of using glutaraldehyde in conjugation reactions is through its ability to react rapidly with hydrazide groups. A molecule-containing hydrazide functionalities or modified to contain them (Chapter 1, Section 4.5) can be conjugated with another molecule containing either amines or hydrazides. Glutaraldehyde will react with the hydrazide groups to form hydrazone linkages. When crosslinking two macromolecules containing multiple sites of conjugation, the multivalent hydrazone bonds are stable enough to create a stable conjugate.

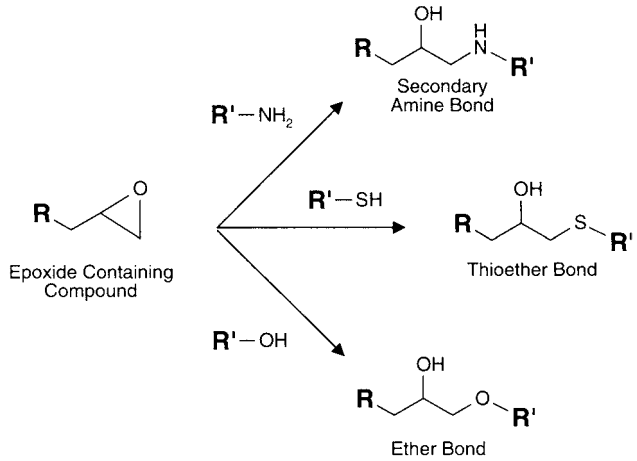


Figure 4.27 Epoxide groups are reactive toward sulfhydryls, amines, and hydroxyls.

If a small molecule is involved, however, reduction of the hydrazone with sodium cyanoborohydride is recommended to produce a leak-resistant bond.

Glutaraldehyde has been used extensively as a homobifunctional crosslinking reagent, especially for antibody–enzyme conjugations (Avrameas, 1969; Avrameas and Ternynck, 1971). To help overcome its tendency to form large-molecular-weight polymers upon crosslinking two proteins, a two-step protocol often is employed. In this regard, one protein first is reacted with glutaraldehyde and purified away from excess reagent. The second protein then is added to effect the conjugate formation. See the introduction to this chapter and Chapter 1, Section 1.2 and Chapter 20, Section 1.2 for additional information on the use of glutaraldehyde and two-step crosslinking procedures.

7. *Bis*-epoxides

Homobifunctional compounds-containing epoxide groups on both ends can be used to crosslink molecules-containing nucleophiles, including amines, sulfhydryls, and hydroxyls. The reaction proceeds with epoxide ring opening to create secondary amine, thioether, or ether bonds with these functional groups (Figure 4.27). During the ring opening process, a β-hydroxy group is created. Hydrolysis of the epoxy function without coupling to a nucleophile yields adjacent hydroxyls that can be oxidized with sodium periodate to create reactive aldehydes (Figure 4.28). Epoxide groups, however, are quite stable in aqueous environments around neutral pH or above. They are reactive at alkaline pH values toward other nucleophilic molecules, while they can be hydrolyzed to a diol at acid pH.

Certain bis-epoxide reagents have been used to activate hydroxylic matrices for coupling ligands-containing amine, sulfhydryl, or hydroxyl groups for affinity chromatography purposes (Hermanson *et al.*, 1992). Conjugation reactions involving proteins also have been done using epoxide crosslinkers, but not to the extent of their use in immobilization.

Bis-epoxy compounds that have been used for crosslinking purposes vary mainly in their chain length, ranging from the 4-carbon bridge of 1,2:3,4-diepoxybutane (Skold, 1983; Kohn *et al.*, 1966), the 6-carbon spacer of 1,2:5,6-diepoxyhexane (Fearnley and Speakman, 1950),

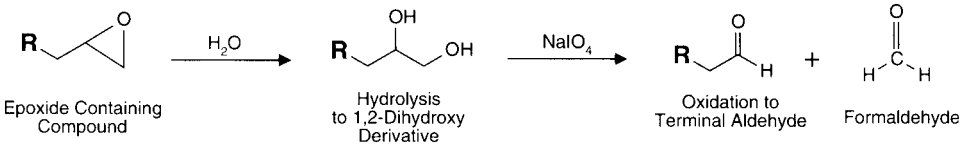
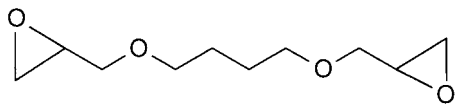


Figure 4.28 Hydrolysis of epoxy groups forms 1,2-dihydroxy derivatives that can be oxidized with periodate to create reactive aldehydes.

the 7-atom bridge of *bis*(2,3-epoxypropyl)ether (Kohn *et al.*, 1966), to the 12-atom spacer of the popular 1,4-(butanediol) diglycidyl ether (discussed below) (Sundberg and Porath, 1974; Porath, 1976). Longer chain polymeric *bis*-epoxide compounds also have been utilized in collagen crosslinking experiments (Murayama *et al.*, 1988).

7.1. 1,4-Butanediol Diglycidyl Ether

The most commonly used homobifunctional epoxide compound is 1,4-butanediol diglycidyl ether. The reagent can react with hydroxyls, amines, or sulfhydryl groups to produce ether, secondary amine, or thioether bonds, respectively. The reaction of the epoxide functionalities with hydroxyls requires high pH conditions, usually in the range of pH 11–12. Amine nucleophiles react at more moderate alkaline pH values, typically needing buffer environments of at least pH 9. Sulfhydryl groups are the most highly reactive nucleophiles with epoxides, requiring a buffered system in the range of only pH 7.5–8.5 for efficient coupling.



1,4-Butanediol Diglycidyl Ether
MW 202.25

1,4-Butanediol diglycidyl ether is a viscous liquid having a density of 1.45 at 20°C. It is a hygroscopic, corrosive compound with a displeasing odor that should be handled with care in a fume hood. Aqueous solutions of the bis-epoxide usually possess a characteristic oily film on their surfaces, indicating the limited solubility of the reagent.

An example of the use of 1,4-butanediol diglycidyl ether for the activation of soluble dextran polymers is given in Chapter 25, Section 2.3. One end of the *bis*-epoxide reacts with the hydroxylic sugar residues of dextran to form ether linkages, which terminate in epoxy functionalities. The epoxides of the activated derivative then can be used to couple additional molecules-containing nucleophilic groups to the dextran backbone.

8. Homobifunctional Hydrazides

Homobifunctional crosslinking agents containing hydrazide groups at both ends can be used to conjugate molecules-containing carbonyl or carboxyl groups. In one scheme, a *bis*-hydrazide compound can be reacted with the carboxylate groups of a protein in the presence of the

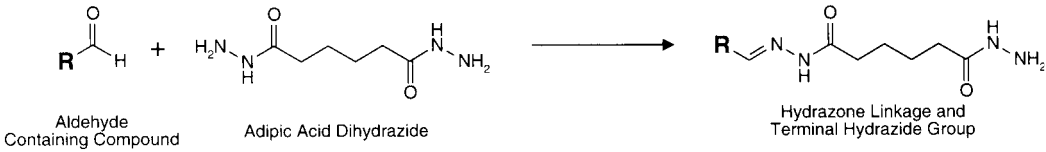


Figure 4.29 ADH spontaneously reacts with aldehydes to form hydrazone linkages.

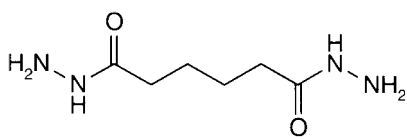
water-soluble carbodiimide, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC), to yield imide linkages-containing terminal alkyl hydrazides. The hydrazide-activated protein then can be used to conjugate with a glycoprotein that had been previously oxidized with sodium periodate to generate reactive aldehyde residues. The resulting hydrazone bonds can be further stabilized by reducing with sodium cyanoborohydride to give the secondary amine linkage.

These techniques have been used to target, detect, or assay glycoproteins in solution or on cell surfaces by using hydrazide-activated enzymes, avidin, or streptavidin (Chapter 23, Section 5) (Bayer and Wilchek, 1990; Bayer *et al.*, 1987a, b, 1990) and to form conjugates with glycoproteins.

Bis-hydrazide-containing molecules also can be used to activate soluble polymeric substances-containing aldehyde groups. For instance, dextran may be periodate oxidized to create numerous formyl functionalities on each molecule. Subsequent reaction with a homobifunctional hydrazide in large excess results in a hydrazide-activated polymer having multivalent-binding capability toward aldehydes or ketones (Chapter 25, Section 2.2). Insoluble support matrices suitable for affinity chromatography have been activated in a similar fashion to create the hydrazide derivative (O'Shannessy and Wilchek, 1990).

8.1. Adipic Acid Dihydrazide

The dihydrazide derivative of adipic acid (Aldrich) is perhaps the most-popular homobifunctional hydrazide compound in use. The reagent provides a 10-atom bridge between crosslinked molecules after conjugation. Adipic dihydrazide (ADH) is a solid that is soluble in aqueous solutions, but may need to be moderately heated to create concentrated solutions. Aldehyde-containing substances may be modified with this reagent to form hydrazone bonds with alkyl hydrazide spacers suitable for reaction with other formyl-containing molecules (Figure 4.29). In this sense, affinity chromatography matrices have been activated with ADH to produce a hydrazide derivative for coupling to aldehyde-containing ligands (O'Shannessy and Wilchek, 1990), enzymes have been modified at available carboxylate groups using an EDC-facilitated reaction to create hydrazide-activated derivatives appropriate for targeting oxidized glycoproteins (Bayer *et al.*, 1987a, b), and the biotin-binding proteins avidin and streptavidin have been



Adipic Acid Dihydrazide
MW 174.2

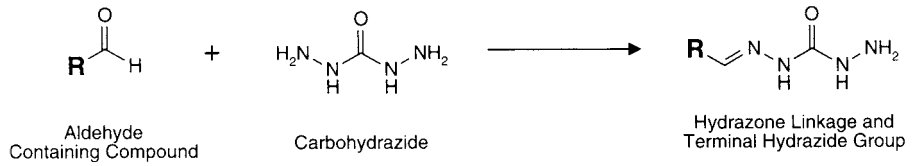


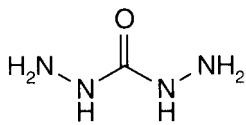
Figure 4.30 Carbohydrazide can be used to transform an aldehyde residue into a hydrazide group.

activated with bis-hydrazides to assay glycoconjugates using biotinylated enzymes (Bayer and Wilchek, 1990; Bayer *et al.*, 1990). The crosslinker also has been utilized to study the carbohydrate portion of yeast acid phosphatase (Kozulic *et al.*, 1984).

Protocols for the use of ADH in the modification of aldehyde or carboxylate functionalities can be found in Chapter 1, Section 4.5 and Chapter 23, Section 5.

8.2. Carbohydrazide

Carbohydrazide (carbonic dihydrazide or 1,3-diaminourea) is a small homobifunctional reagent containing reactive hydrazide groups on both ends. Its lack of an internal aliphatic bridge, as found in ADH, gives the compound excellent solubility characteristics in aqueous solutions. Carbohydrazide is freely soluble in water, but practically insoluble in ethanol and other organic solvents. The two hydrazide functional groups of the molecule can react with aldehyde or ketone groups to form hydrazone linkages. When reacted in excess with a molecule-containing carbonyl groups, carbohydrazide modification results in short derivatives terminating in available hydrazides (Figure 4.30). The compound has been used to modify microplate wells that have been graft polymerized with glycidyl methacrylate to form surfaces that would couple antibodies through their carbohydrate portions (Allmer *et al.*, 1990; Brillhart and Ngo, 1991). Although its use for protein modification has not been realized, carbohydrazide may be a superior alternative to ADH due to its hydrophilicity. Its only disadvantage may be in its shorter bridge (5-atom spacer as opposed to ADH's 10-atom bridge).



Carbohydrazide
MW 90.09

A protocol for the use of carbohydrazide in the modification of aldehyde or carboxylate functional groups can be found in Chapter 1, Section 4.5.

9. *Bis*-diazonium Derivatives

Diazonium groups react with active hydrogens on aromatic rings to give covalent diazo bonds. Generation of a diazonium-reactive group usually is done from an aromatic amine by reaction

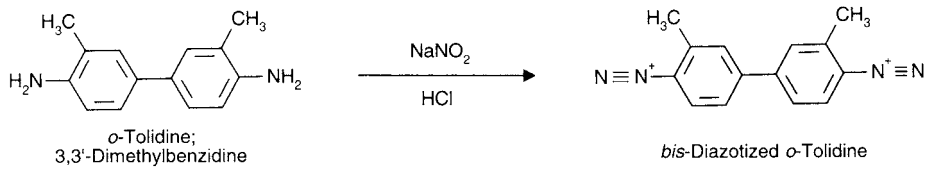


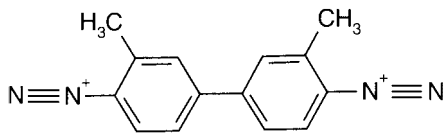
Figure 4.31 Reaction of *o*-tolidine with sodium nitrite in the presence of HCl yields a highly reactive diazo derivative.

with sodium nitrite under acidic conditions at 0°C (see Chapter 1, Section 4.3 and Chapter 2, Section 6.1). The highly reactive and unstable diazonium is reacted immediately with an active-hydrogen-containing compound at pH 8–10. In general, at pH 8.0 the diazonium group will react principally with histidine residues, attacking the electron rich nitrogens of the imidazole ring. At higher pH, the phenolic side chain of tyrosine groups can be modified. The reaction proceeds by electrophilic attack of the diazonium group toward the electron rich points on the target molecules. Phenolic compounds are modified at positions *ortho* and *para* to the aromatic hydroxyl group. For tyrosine side chains, only the *ortho* modification is available.

Bis-diazonium compounds are useful in crosslinking molecules containing no other convenient functional groups such as amines, carboxylates, or sulfhydryls. Conjugations done using these compounds usually create deeply colored products characteristic of the diazo bonds. Occasionally, the conjugated molecules may turn dark brown or even black. The diazo linkages are reversible by addition of 0.1 M sodium dithionite in 0.2 M sodium borate, pH 9.0. Upon cleavage, the color of the complex is lost.

9.1. *o*-Tolidine, Diazotized

o-Tolidine, or 3,3′-dimethylbenzidine, is a *bis*-aromatic-amine-containing compound that can be readily diazotized to a homobifunctional diazonium crosslinker by reaction with sodium nitrite (Figure 4.31). The reagent is typically used in a one-step conjugation reaction wherein two active-hydrogen-containing molecules are crosslinked through the addition of *o*-tolidine immediately after it has been diazotized under acidic conditions by reacting with sodium nitrite (Figure 4.32). pH adjustment to alkaline conditions after diazonium formation rapidly causes crosslinking to occur. The diazotized form of *o*-tolidine must be used quickly due to its instability in aqueous solutions. The reagent has been used to couple active-hydrogen-containing haptens to carrier proteins to form immunogens suitable for the production of antibodies (Chapter 19, Section 6.1).



bis-Diazotized *o*-Tolidine

o-Tolidine is a benzidine derivative that should be considered a potential carcinogen. Handling should be done with proper safety precautions and with the use of a fume hood to

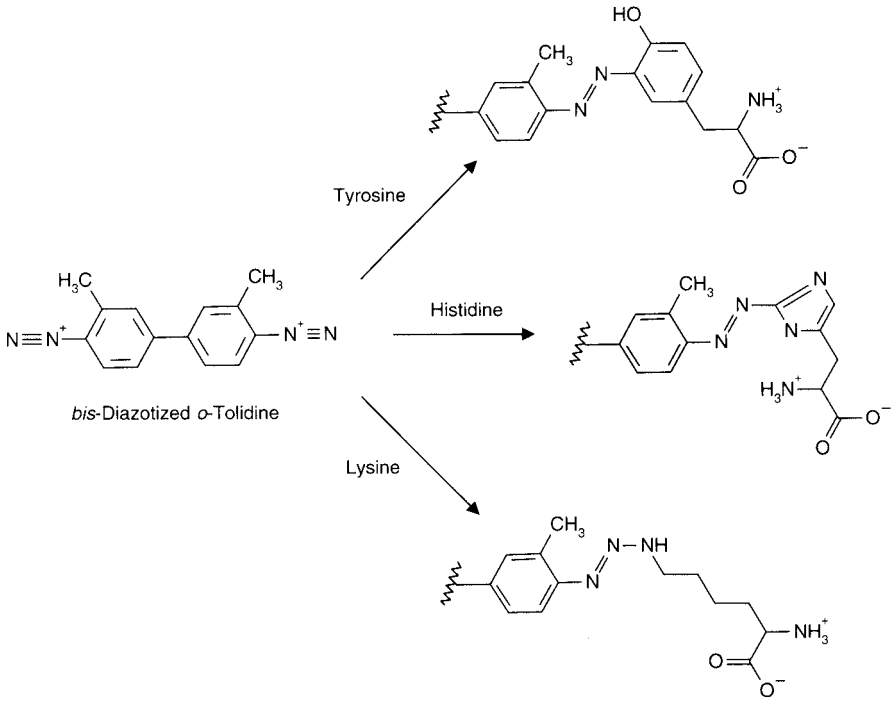
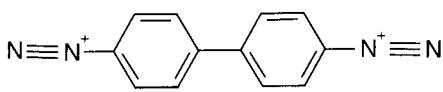


Figure 4.32 *Bis*-diazotized tolidine can form crosslinks with proteins through available tyrosine, histidine, or lysine residues.

avoid breathing in any dust particles. The reagent is sparingly soluble in water, but is more soluble under the dilute acidic conditions necessary for activation to a diazonium derivative.

9.2. *Bis*-diazotized Benzidine

Benzidine, or *p*-diaminodiphenyl, may be diazotized with sodium nitrite to form a homobifunctional diazonium crosslinking agent useful in conjugating active-hydrogen-containing molecules (Figure 4.33). The coupling reaction proceeds via electrophilic attack on atoms containing extractable hydrogens. Particularly reactive are the phenolic side chains of tyrosine residues and the imidazole rings of histidine groups.



Bis-Diazotized Benzidine

Benzidine is a known carcinogen and should be handled with extreme caution (Fourth Annual Report on Carcinogens; NTP 85-002, 1985, p. 37). The solid and its vapors may be rapidly absorbed through skin. Protective clothing and the use of a fume hood are

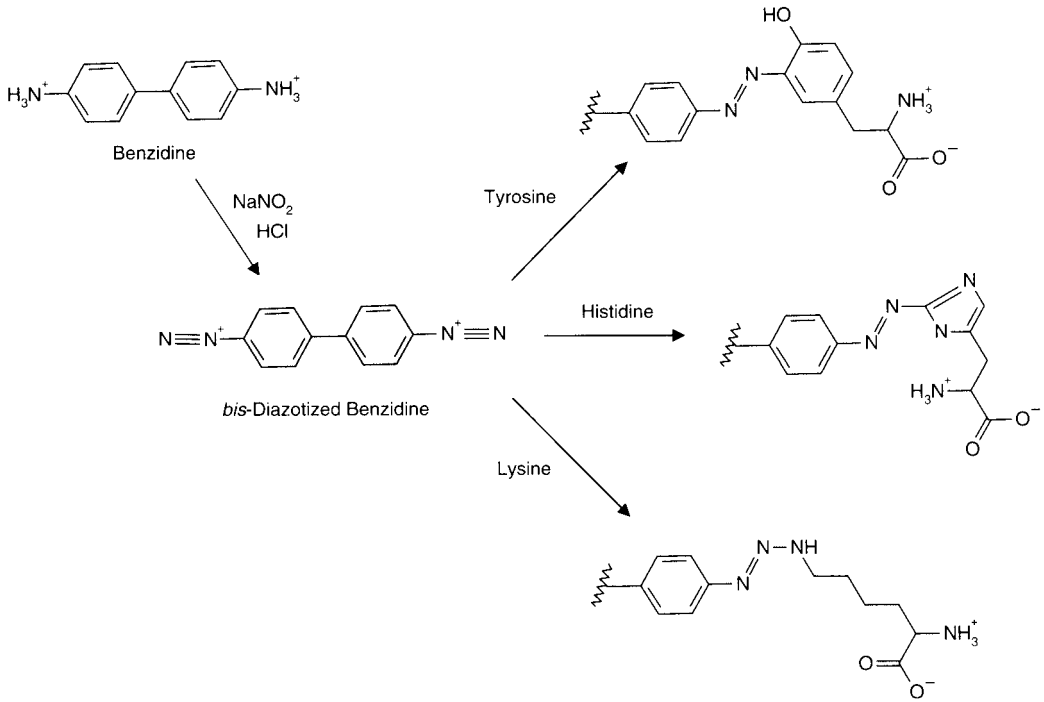


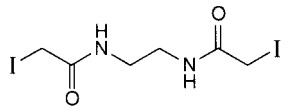
Figure 4.33 Benzidine can be diazotized with sodium nitrite and HCl for reaction with proteins through their tyrosine, histidine, or lysine side-chain groups.

recommended. The compound is only sparingly soluble in water as the free base. The dihydrochloride form, however, is soluble in water and ethanol.

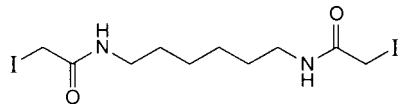
Bis-diazotized benzidine has been used to create active-hydrogen-reactive spacer arms on chromatographic matrices (Silman *et al.*, 1966; Lowe and Dean, 1971). The compound may be used similarly to *o*-tolidine for the conjugation of active-hydrogen-containing molecules (see Section 9.1, this chapter, and Chapter 2, Section 6.1).

10. *Bis*-alkyl Halides

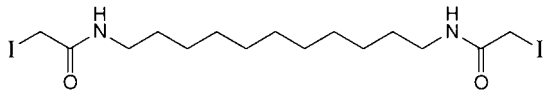
Homobifunctional reagents containing reactive halogen groups on both ends are capable of crosslinking sulfhydryl, amine, or histidine-containing molecules by nucleophilic substitution. Three forms of activated halogen functionalities can be used to create these reagents: haloacetyl derivatives (see Chapter 1, Section 5.2), benzyl halides that react through a resonance activation process with the neighboring benzene ring, and alkyl halides that possess the halogen β to a nitrogen or sulfur atom, as in *N*- and *S*-mustards. Haloacetyl compounds typically are iodo or bromo derivatives, the simplest of which are 1,3-dibromoacetone (Husain and Lowe, 1968) and various iodoacetyl derivatives of short diamine alkyl spacers (Ozawa, 1967) (Figure 4.34). Benzyl halides also are usually iodo or bromo derivatives, whereas the halo-mustards mainly employ chloro and bromo forms (Figure 4.35).



N,N'-Ethylene-bis(iodoacetamide)

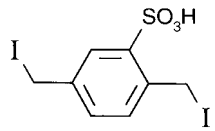


N,N'-Hexamethylene-bis(iodoacetamide)

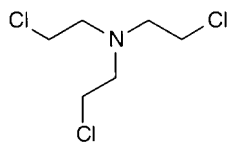


N,N'-Undecamethylene-bis(iodoacetamide)

Figure 4.34 Several varieties of iodoacetylated diamine compounds have been investigated for crosslinking proteins through sulfhydryl groups.



α,α'-Diiodo-p-xylene sulfonic acid



TCEA;
Tri(2-chloroethyl)amine

Figure 4.35 Benzyl halides and halomustards can be used as crosslinking agents reactive toward sulfhydryl groups.

Reactive halogen crosslinkers are mainly specific for sulfhydryl groups at physiological pH, however at more alkaline pH values they can readily cross-react with amines and the imidazole nitrogens of histidine residues. Some reactivity with hydroxyl-containing compounds also may be realized, particularly with dichloro-*s*-triazine derivatives under alkaline conditions.

Most of the *bis*-alkyl halides referenced in the literature are unavailable commercially, and therefore must be synthesized. Some key references to the preparation and use of these compounds for the crosslinking of sulfhydryl-containing proteins and other molecules include Goodlad (1957), Segal and Hurwitz (1976), Ewig and Kohn (1977), Wilchek and Givol (1977), Prestayko *et al.* (1981), Luduena *et al.* (1982), Hiratsuka (1988), and Aliosman *et al.* (1989).

5

Heterobifunctional Crosslinkers

Heterobifunctional conjugation reagents contain two different reactive groups that can couple to two different functional targets on proteins and other macromolecules (Figure 5.1). For example, one part of a crosslinker may contain an amine-reactive group, while another portion may consist of a sulfhydryl-reactive group. The result is the ability to direct the crosslinking reaction to selected parts of target molecules, thus garnering better control over the conjugation process.

Heterobifunctional reagents can be used to crosslink proteins and other molecules in a two- or three-step process that limits the degree of polymerization often obtained using homobifunctional crosslinkers (Chapter 1, Section 1.2 and Chapter 4, Section 2.2). In a typical conjugation scheme, one protein is modified with a heterobifunctional compound using the crosslinker's most reactive or most labile end. The modified protein then is purified from excess reagent by gel filtration or rapid dialysis. Most heterobifunctionals contain at least one reactive group that displays extended stability in aqueous environments, therefore allowing purification of an activated intermediate before adding the second molecule to be conjugated. For instance, an *N*-hydroxysuccinimide (NHS) ester–maleimide heterobifunctional (e.g., see Section 1.3, this chapter) can be used to react with the amine groups of one protein through its NHS ester end (the most labile functionality), while preserving the activity of its maleimide functionality. Since the maleimide group has greater stability in aqueous solution than the NHS ester group, a maleimide-activated intermediate may be created. After a quick purification step, the maleimide end of the crosslinker then can be used to conjugate to a sulfhydryl-containing molecule.

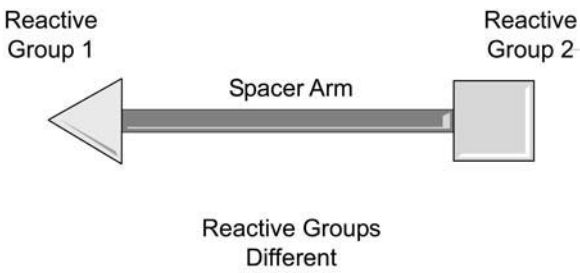


Figure 5.1 The general design of a heterobifunctional crosslinking agent includes two different reactive groups at either end and an organic cross-bridge of various length and composition. The cross-bridge may be constructed of chemically cleavable components for selective disruption of conjugates.

Such multi-step protocols offer greater control over the resultant size of the conjugate and the molar ratio of components within the crosslinked product. The configuration or structure of the conjugate can be regulated by the degree of initial modification of the first protein and by adjusting the amount of second protein added to the final conjugation reaction. Thus, low- or high-molecule-weight conjugates may be obtained to better fashion the product toward its intended use.

Heterobifunctional crosslinking reagents also may be used to site-direct a conjugation reaction toward particular parts of target molecules. Amines may be coupled on one molecule while sulfhydryls or carbohydrates are targeted on another molecule. Directed coupling often is important in preserving critical epitopes or active sites within macromolecules. For instance, antibodies may be coupled to other proteins while directing the crosslinking reaction away from the antigen binding sites, thus maximizing antibody activity in the conjugate.

Heterobifunctional reagents containing one photoreactive end may be used to insert non-selectively into target molecules by UV irradiation. Ligands having specific affinity toward a receptor may be labeled with a photoreactive crosslinker, allowed to interact with its target, and then photolyzed to permanently label the receptor at its binding site. The photoreactive group is stable until exposed to high-intensity light at UV wavelengths. Photoaffinity labeling techniques are an important investigative tool for determining binding site characteristics.

The third component of all heterobifunctional reagents is the cross-bridge or spacer that ties the two reactive ends together. Crosslinkers may be selected based not only on their reactivities, but on the length and type of cross-bridge they possess. Some heterobifunctional families differ solely in the length of their spacer. The nature of the cross-bridge also may govern the overall hydrophilicity of the reagent. For instance, polyethylene glycol (PEG)-based cross-bridges create hydrophilic reagents that provide water solubility to the entire heterobifunctional compound (see Chapter 18). A number of heterobifunctionals contain cleavable groups within their cross-bridge, lending greater flexibility to the experimental design. A few crosslinkers contain peculiar cross-bridge constituents that actually affect the reactivity of their functional groups. For instance, it is known that a maleimide group that has an aromatic ring immediately next to it is less stable to ring opening and loss of activity than a maleimide that has an aliphatic ring adjacent to it. In addition, conjugates destined for use *in vivo* may have different properties depending on the type of spacer on the associated crosslinker. Some spacers may be immunogenic and cause specific antibody production to occur against them. In other instances, the half-life of a conjugate *in vivo* may be altered by choice of cross-bridge, especially when using cleavable reagents.

The following heterobifunctional reagents are organized according to their reactivities. The majority are commercially available and well documented in the literature as to their properties. Additional heterobifunctional compounds are described in Chapter 17 (Chemoselective Ligation; Bioorthogonal Reagents) and Chapter 18 (Discrete PEG Reagents).

1. Amine-Reactive and Sulfhydryl-Reactive Crosslinkers

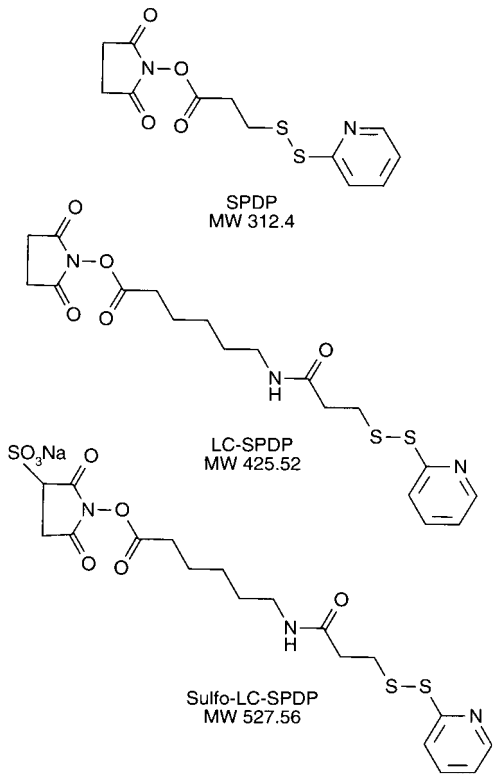
Perhaps the most popular heterobifunctional reagents are those which contain amine-reactive and sulfhydryl-reactive ends. The amine-reactive group is usually an active ester, most often an NHS ester, while the sulfhydryl-reactive portion may be one of several different functional groups. The amine-reactive end of these crosslinkers is typically an acylating agent possessing a good leaving group that can undergo nucleophilic substitution to form an amide bond with primary amines. The sulfhydryl-reactive portion, by contrast, is usually an alkylating agent that is

capable of creating either thioether or disulfide linkages with sulfhydryl-containing molecules. Depending on the chemistry chosen, linkages with a sulfhydryl-containing molecule may be either permanent covalent bonds or reversible disulfide bonds that can be cleaved by use of a suitable disulfide reductant.

The active ester chemistry of the amine-reactive end of these crosslinkers is characteristically the most labile functional group, being susceptible to rapid hydrolysis under the aqueous conditions of a conjugation reaction. The sulfhydryl-reactive group, however, is usually much more stable to breakdown in aqueous environments. Therefore, these reagents typically are used in multi-step conjugation protocols wherein one protein or molecule first is modified through its amines to yield a sulfhydryl-reactive intermediate. After removal of excess crosslinker by gel filtration, a second protein or molecule containing a sulfhydryl group is added to effect the final conjugation. The stability of the sulfhydryl-reactive end of these crosslinkers allows greater control over the crosslinking process than is possible with single-step procedures.

1.1. SPDP, LC-SPDP, and Sulfo-LC-SPDP

SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate, is one of the most popular heterobifunctional crosslinking agents available. The activated NHS ester end of SPDP reacts with amine



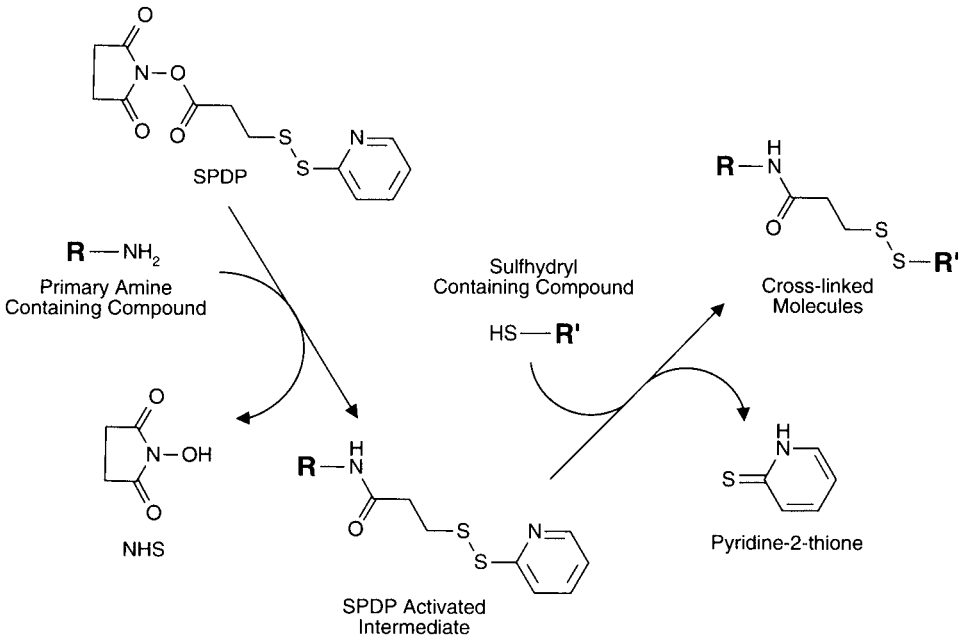


Figure 5.2 SPDP can react with amine-containing molecules through its NHS ester end to form amide bonds. The pyridyl disulfide group then can be coupled to a sulfhydryl-containing molecule to create a cleavable disulfide bond.

groups in proteins and other molecules to form an amide linkage. The 2-pyridyldithiol group at the other end reacts with sulfhydryl residues to form a disulfide linkage with sulfhydryl-containing molecules (Carlsson *et al.*, 1978) (Figure 5.2). The crosslinker is used extensively to form enzyme conjugates for use in immunoassays or in labeled DNA probe techniques. It also is frequently used for the preparation of immunotoxin conjugates for *in vivo* administration (Chapter 21, Section 2.1 and Chapter 27, Section 2.4). In addition, the reagent is effective in creating sulfhydryls on proteins and other molecules (Chapter 1, Section 4.1). Once modified with SPDP, a protein can be treated with dithiothreitol (DTT) (or another disulfide reducing agent) to release the pyridine-2-thione leaving group and form a free sulfhydryl. The terminal —SH group then can be used to conjugate with any crosslinking agents containing sulfhydryl-reactive groups, such as maleimide or iodoacetyl (both for covalent conjugation) or 2-pyridyldithiol groups (for reversible conjugation).

There are three forms of SPDP analogs currently available commercially (Thermo Fisher): the standard SPDP, a long-chain version designated LC-SPDP, and a water-soluble, sulfo-NHS form also containing an extended chain, called sulfo-LC-SPDP. Both the standard SPDP and the LC-SPDP are insoluble in aqueous solutions and must be first solubilized in DMSO prior to addition to the reaction solution. The sulfo-LC-SPDP may be solubilized directly in water or buffer. The long-chain versions extend the length of the crosslinker for those applications that require greater accessibility to react with sterically hindered functional groups. Since many sulfhydryl residues are found below the surface of a protein structure in more hydrophobic domains, the longer spacer arm of the LC versions may be more effective in conjugations with

these groups. The deficiency in having long aliphatic spacers, however, is that the reagent becomes much more hydrophobic. Care should be taken not to over-modify proteins in order to avoid the potential for precipitation.

SPDP or its analogs have been used in many conjugation applications, including the preparation of peptide-based immunoconjugates for prostate cancer therapy (Rege *et al.*, 2007), the preparation of antibody conjugates for a time-resolved fluorescence assay system (Liang *et al.*, 2007), and in the study of bone morphogenetic protein type-II receptor in pulmonary hypertension (Reynolds *et al.*, 2007).

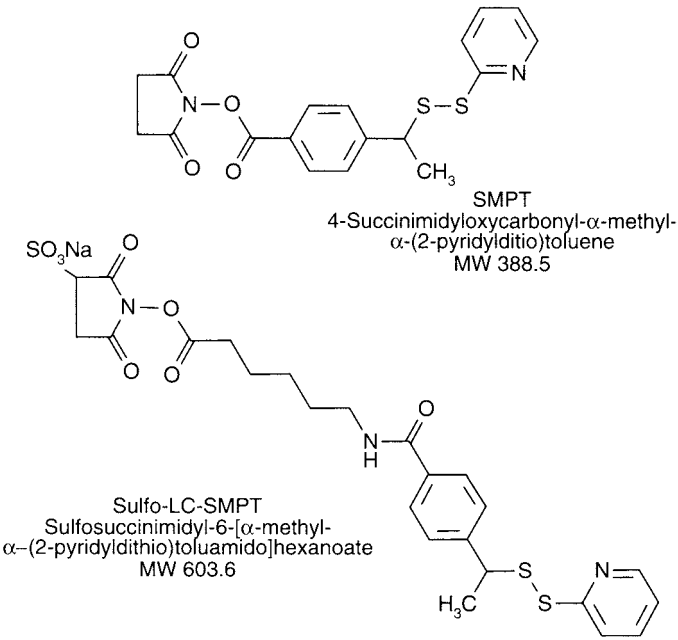
The following procedure is a suggested multi-step protocol involving the activation of one protein by modification of its amines through the NHS ester end of SPDP, purification of this active intermediate, and subsequent addition of a sulfhydryl-containing molecule for conjugation via the remaining pyridyl disulfide group.

Protocol

1. Dissolve a protein or macromolecule containing primary amines at a concentration of 10 mg/ml in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Other non-amine-containing buffers such as borate, HEPES, and bicarbonate also may be used in this reaction. Avoid sulfhydryl-containing components in the reaction mixture as these will react with the pyridyl disulfide end of SPDP. The effective pH for the NHS ester modification reaction is in the range of 7–9, but hydrolysis will increase at the higher end of this range.
2. Dissolve SPDP at a concentration of 6.2 mg/ml in DMSO (makes a 20 mM stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 mM solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to adding an aliquot to the thiolation reaction. In this case, prepare a 10 mM solution of sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of protein will be modified, the solid may be added directly to the reaction mixture without preparing a stock solution in water to allow accurate weighing of sulfo-LC-SPDP.
3. Add 25 µl of the stock solution of either SPDP or LC-SPDP in DMSO to each ml of the protein to be modified. If sulfo-LC-SPDP is used, add 50 µl of the stock solution in water to each ml of protein solution.
4. Mix and react for at least 30 minutes at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis or gel filtration using 50 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. Alternatively, centrifugal spin columns containing a desalting resin may be used for rapid purification (Thermo Fisher).
6. Add a sulfhydryl-containing protein or other molecule to the purified SPDP-modified protein to effect the conjugation reaction. Molecules lacking available sulfhydryl groups may be modified to contain them by a number of methods (Chapter 1, Section 4.1). The amount of this second protein added to the reaction should be governed by the desired molar ratio of the two proteins in the final conjugate. The conjugation reaction should be done in the presence of at least 10 mM EDTA to prevent metal-catalyzed sulfhydryl oxidation.

1.2. SMPT and Sulfo-LC-SMPT

SMPT, succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene, is a heterobifunctional crosslinking agent that contains an amine-reactive NHS ester on one end and a sulfhydryl-reactive pyridyl disulfide group on the other end. SMPT is therefore an analog of SPDP that differs only in its cross-bridge, which contains an aromatic ring and a hindered disulfide group (Thorpe *et al.*, 1987; Ghetie *et al.*, 1990). The spacer arm of SMPT is slightly longer than SPDP (11.2 Å versus 6.8 Å), but the presence of the benzene ring and an α-methyl group adjacent to the disulfide sterically hinders the structure sufficiently to provide increased half-life of conjugates *in vivo*.



Conjugation reactions done using SMPT often proceed by a multi-step protocol involving modification of one protein through its amine groups to create a pyridyl disulfide-activated intermediate. Since SMPT is not soluble in water, the reagent first is solubilized in DMF or DMSO and an aliquot of this stock solution added to the reaction. The NHS ester end of the reagent reacts with ε- and N-terminal amine groups to create stable amide linkages. After removal of excess crosslinker by gel filtration or dialysis, a second protein containing a sulfhydryl group is added to effect the final conjugation (Figure 5.3). The resultant protein–protein crosslink contains a disulfide bond that is susceptible to cleavage by reduction, although more slowly due to the hindered nature of the cross-bridge.

SMPT often is used for the preparation of immunotoxin conjugates that contain a monoclonal antibody directed against some cell-surface antigen (usually a tumor-associated antigen) crosslinked to a protein toxin molecule. It has been shown that a cleavable linkage between the antibody and toxin molecules helps to assure a potent immunotoxin (Lambert *et al.*, 1985). Increased cytotoxicity typically is observed for immunotoxin conjugates containing cross-bridge

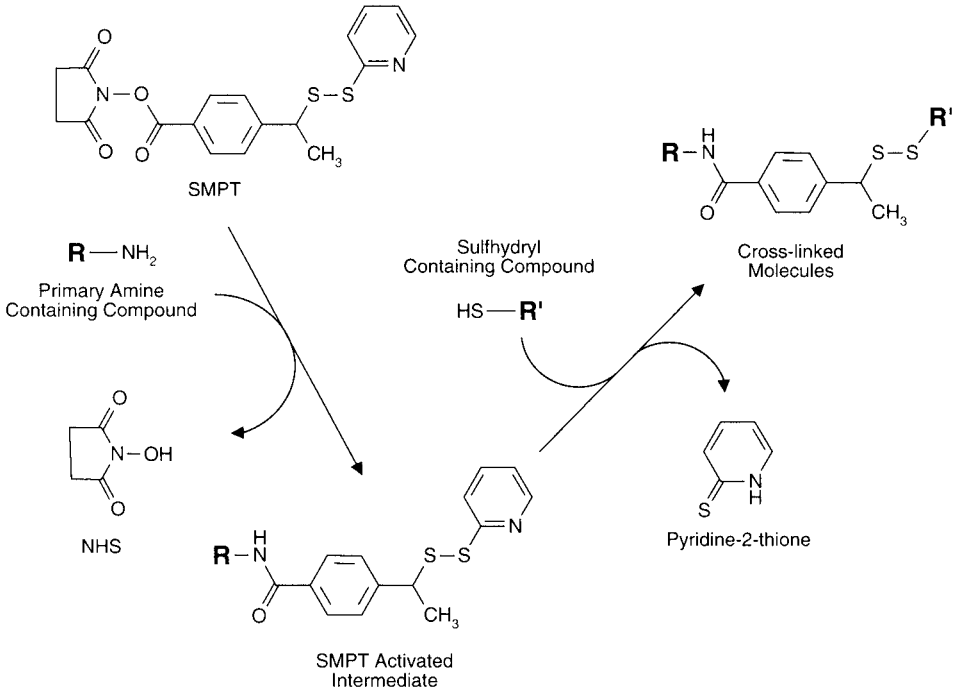


Figure 5.3 SMPT can form crosslinks between an amine-containing molecule and a sulfhydryl-containing compound through amide and disulfide linkages, respectively. The hindered nature of the disulfide group provides better stability toward reduction and cleavage.

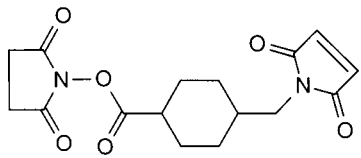
disulfides as opposed to non-cleavable linkages. Cleavability presumably facilitates the release of the toxin from the antibody after the conjugate has bound to the cell surface. However, the disulfide bonds formed from some crosslinkers, such as SPDP, are readily reduced and cleaved *in vivo*—often before they reach their target. The hindered disulfide of SMPT has distinct advantages in this regard. Thorpe *et al.* (1987) showed that SMPT conjugates had approximately twice the half-life *in vivo* as SPDP conjugates.

A water-soluble analog of SMPT, called sulfo-LC-SMPT, or sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate, is available from Thermo Fisher. The sulfo-NHS ester end of the reagent provides the water solubility due to the negative charge of the sulfonate group. While sulfo-LC-SMPT has the same chemical reactivity as SMPT, its cross-bridge contains an additional 6-aminocaproic acid spacer providing a 20 Å crosslink as opposed to the 11.2 Å length of SMPT. The reactivity and use of sulfo-LC-SMPT is essentially the same as that of SMPT, except that the reagent may be added directly to aqueous reaction media or pre-dissolved in water. A stock solution made in water should be used immediately to prevent extensive NHS ester hydrolysis.

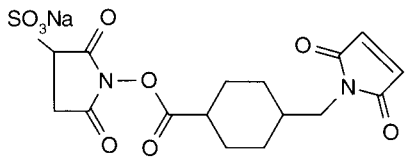
SMPT or sulfo-LC-SMPT has been used to develop conjugates for *in vivo* delivery of siRNA to hepatocytes (Rozema *et al.*, 2007), in preparing an anti-CD25-immunotoxin conjugate (Mielke *et al.*, 2007), and in preparing conjugates for selective depletion of donor lymphocytes in stem cell transplantation (Solomon *et al.*, 2005).

1.3. SMCC and Sulfo-SMCC

SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, is a heterobifunctional reagent with significant utility in crosslinking proteins, particularly in the preparation of antibody–enzyme and hapten–carrier conjugates (Hashida and Ishikawa, 1985; Dewey *et al.*, 1987). In fact, it may be the most popular crosslinker ever designed for protein conjugation purposes. The NHS ester end of the reagent can react with primary amine groups on proteins to form stable amide bonds. The maleimide end of SMCC is specific for coupling to sulfhydryls when the reaction pH is in the range of 6.5–7.5 (Smyth *et al.*, 1964) (Figure 5.4).



Sulfo-SMCC;
Succinimidyl 4-(N-maleimidomethyl)-
cyclohexane-1-carboxylate
MW 334.33
11.6 Å



Sulfo-SMCC;
Sulfosuccinimidyl 4-(N-maleimidomethyl)-
cyclohexane-1-carboxylate
Water Soluble
MW 436.37
11.6 Å

At pH 7, the reaction of the maleimide group with sulfhydryls proceeds at a rate 1,000 times greater than its reaction with amines. At more alkaline pH values, however, its reaction with amines becomes more evident. The maleimide end also may undergo hydrolysis to an open maleamic acid form that is unreactive toward sulfhydryls. Hydrolysis may occur after sulfhydryl coupling to the maleimide, as well. This ring-opening reaction typically happens faster at higher pH values. However, the maleimide group of SMCC displays unusual stability up to pH 7.5. The increased stability of SMCC's maleimide group may be due to it not being attached directly to an aromatic ring structure. By contrast, some maleimide-containing reagents, such as *N*,*N*′-*o*-phenylenedimaleimide and *N*,*N*′-oxydimethylenedimaleimide are far less stable under these conditions. Reportedly, only 4 percent of the maleimide groups of SMCC will decompose at neutral pH within 2 hours at 30°C (Ishikawa *et al.*, 1983a, b). For this reason, proteins may be modified with SMCC to form relatively long-lived, maleimide-activated intermediates. The SMCC derivative then may be freeze-dried to provide a stock preparation of sulfhydryl-reactive protein.

SMCC frequently is used to prepare hapten–carrier or antibody–enzyme conjugates. In both applications, one of the molecules is activated (usually the carrier or the enzyme) with the

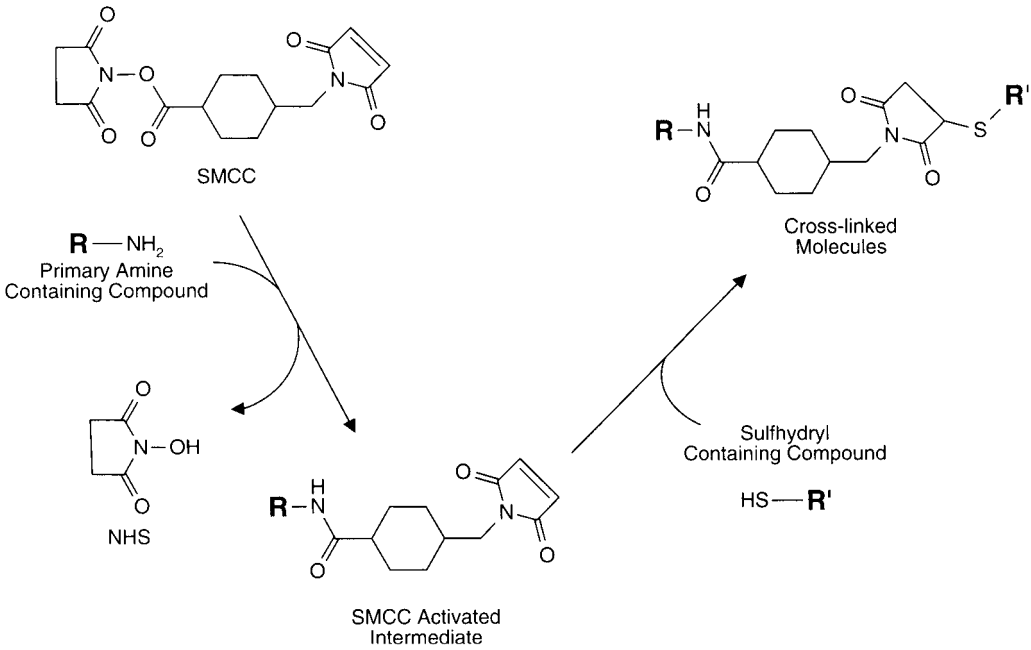


Figure 5.4 SMCC reacts with amine-containing molecules to form stable amide bonds. Its maleimide end then may be conjugated to a sulfhydryl-containing compound to create a thioether linkage.

crosslinker, purified to remove excess reagents, and then mixed with the sulfhydryl-containing second molecule to make the final conjugate. Published applications using SMCC are numerous, but include conjugation of glucose oxidase to rabbit antibodies (Yoshitake *et al.*, 1979), crosslinking Fab′ fragments to horseradish peroxidase (Imagawa *et al.*, 1982; Yoshitake *et al.*, 1982a, b; Ishikawa *et al.*, 1983a, b; Uto *et al.*, 1991), coupling anti-digoxin $F(ab')_2$ fragments to β-galactosidase (Freytag *et al.*, 1984a, b), preparing conjugates of alkaline phosphatase and human IgG $F(ab')_2$ fragments (Mahan *et al.*, 1987), and use in the preparation of immunogens (Peeters *et al.*, 1989). SMCC also has been used to prepare antibody–drug conjugates targeted to CD79 for treatment of non-Hodgkin lymphoma (Polson *et al.*, 2007), to make antibody-conjugated, radiolabeled carbon nanotubes for tumor targeting (McDevitt *et al.*, 2007), and in the preparation of peptide–ovalbumin conjugates to study nucleus-to-cytoplasm shuttling of human aci-reductone dioxygenase (Gotoh *et al.*, 2007).

Since SMCC is a water-insoluble crosslinker, it must be dissolved first in organic solvent (DMSO or DMF) before adding it to a protein to be modified. In some cases, addition of even a small amount of organic solvent to a protein solution may be detrimental to activity. To be safe, no more than 10–20 percent solvent should be present in the aqueous reaction medium.

Sulfo-SMCC, sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, is a water-soluble analog of SMCC that possesses a negatively charged sulfonate group on it NHS ring. The charge gives just enough polarity to the molecule to provide water solubility at a level of at least 10 mg/ml at room temperature. This allows direct addition of the reagent to reaction mixtures without prior dissolution in organic solvent. The crosslinker is known to be soluble at a concentration of at least 10 mM in the following buffers: (a) 50 mM sodium acetate,

pH 5.0, (b) 50 mM sodium borate, pH 7.6, and (c) 0.1 M sodium phosphate, pH 6–7.5. Aqueous stock solutions may be prepared using sulfo-SMCC, but these should be dissolved rapidly and used immediately to prevent extensive loss of sulfo-NHS coupling ability due to hydrolysis. Concentrated aqueous stock solutions (up to about 50 mg/ml) may be made by heating for a few minutes under hot running water. Quickly cool to room temperature before using. However, to avoid the potential of activity loss by hydrolysis, even sulfo-SMCC may be dissolved in DMSO prior to adding a small aliquot to an aqueous reaction.

The following is a generalized protocol for the activation of a protein with sulfo-SMCC with subsequent conjugation to a sulfhydryl-containing second molecule or protein. Specific examples of the use of this crosslinker to make antibody–enzyme or hapten–carrier conjugates may be found in Chapter 20, Section 1.1 and Chapter 19, Section 5, respectively.

Protocol

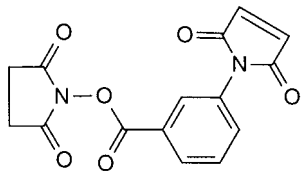
1. Dissolve 10 mg of a protein or other macromolecule to be activated with sulfo-SMCC in 1 ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. Weigh out 2 mg of sulfo-SMCC and add it to the above solution. Mix gently to dissolve. To aid in measuring the exact quantity of crosslinker, a concentrated stock solution may be made in water (or DMSO) and an aliquot equal to 2 mg transferred to the reaction solution. If a stock solution is made, it should be dissolved rapidly and used immediately to prevent extensive hydrolysis of the active ester. As a general guideline of addition for a particular protein activation, the use of a 40- to 80-fold molar excess of crosslinker over the amount of protein present usually results in good activation levels.
3. React for 1 hour at room temperature with periodic mixing.
4. Immediately purify the maleimide-activated protein by applying the reaction mixture to a desalting column packed with a desalting resin. The use of a centrifugal spin column may provide faster separations (Thermo Fisher). Do not dialyze the solution, since the maleimide activity will be lost over the time course required to complete the operation. To obtain good separation between the protein peak (eluting first) and the peak representing excess reagent and reaction by-products (eluting second), the applied sample size should be no more than 8 percent of the column bed volume. If complete separation of the activated protein from excess crosslinker is not obtained, then the maleimide content contributed from contaminating crosslinker may prevent subsequent conjugate formation. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2. Collect 1 ml fractions and pool the peak containing the protein. At this point, the maleimide-activated protein may be used immediately in a conjugation reaction with a sulfhydryl-containing protein or other molecule or freeze-dried to preserve the maleimide activity.
5. To effect the conjugation reaction, mix the maleimide-activated protein at the desired molar ratio with a sulfhydryl-containing molecule dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2. The purified protein from step 4 may be concentrated if necessary using centrifugal concentrators, but this should be done quickly to avoid extensive loss of activity. The molar ratio of addition depends on the desired conjugate to be obtained. For instance, if coupling a sulfhydryl-containing small molecule to a protein, the molecule should be added in excess to the amount of maleimide activity present on the protein. In such a case, a 10- to 100-fold molar excess may be appropriate (see Chapter 19, Section 5). However, if preparing protein–protein conjugates, as in the case of antibody–enzyme

conjugates, the ratio of maleimide-activated protein to the sulfhydryl-containing protein is a matter of choice. Often, when coupling enzymes to antibodies, the enzyme is in molar excess over the antibody (see Chapter 20, Section 1.1). Typical molar ratios of enzyme-to-antibody can range from 2:1 to 7:1.

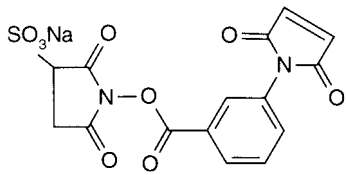
6. React for 2–24 hours at room temperature or 4–24 hours at 4°C.
7. The conjugate may be isolated by gel filtration if the molecular weight of the complex is sufficiently different from that of the unconjugated molecules.

1.4. MBS and Sulfo-MBS

MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester, is a heterobifunctional crosslinking agent containing an NHS ester on one end and a maleimide group on the other end. The NHS ester can react with primary amines in proteins and other molecules to form stable amide bonds, while the maleimide end nearly exclusively reacts with sulfhydryl groups to create stable thioether linkages (Figure 5.5). These characteristics allow highly controlled conjugation reactions to be done with MBS using two- or three-step processes. In this sense, the NHS ester end of the reagent typically is reacted with the first protein to be crosslinked, forming a maleimide-activated intermediate. The maleimide group is more stable to break down by hydrolysis than the NHS ester, so the activated intermediate can be quickly purified from excess crosslinker and reaction by-products before adding it to the sulfhydryl-containing second molecule. However, due to the aromatic ring adjacent to its maleimide functional group, MBS displays less stability toward maleimide ring opening than SMCC (Section 1.3, this chapter). Unlike SMCC, MBS is therefore not recommended for preparing freeze-dried, maleimide-activated proteins, since during the processing necessary to purify and stabilize the derivative much activity can be lost by hydrolysis.



MBS;
m-Maleimidobenzoyl-N-hydroxy-
succinimide ester
MW 314.2
9.9 Å



Sulfo-MBS;
m-Maleimidobenzoyl-N-hydroxy-
sulfosuccinimide ester
MW 416.24
9.9 Å

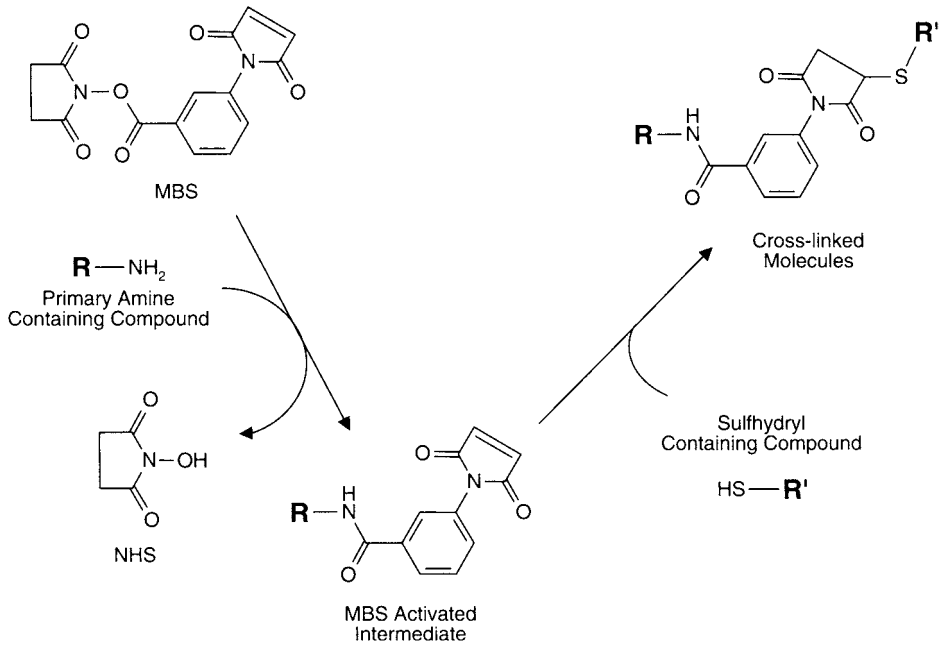


Figure 5.5 The two-step conjugation procedure for the MBS crosslinking of an amine-containing molecule with a sulfhydryl-containing molecule.

MBS contains a benzoic acid derivative as its cross-bridge, thus lending considerable hydrophobicity to the entire molecule. Since the reagent is water-insoluble, it must be first dissolved in organic solvent before adding it to an aqueous reaction medium. Making a concentrated stock solution of MBS in DMF or DMSO allows transfer of a small amount to a conjugation reaction (total concentration of the organic solvent should not exceed 10 percent in the reaction buffer). When these solvents are used, a micro-emulsion typically is formed in the aqueous solution, which provides crosslinker efficiently to the conjugating species. The reagent also is readily permeable to membrane structures due to its hydrophobic nature.

Sulfo-MBS, *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester, is a water-soluble analog of MBS that contains a negatively charged sulfonate group on its NHS ring (Martin and Papahadjopoulos, 1982; Aithal *et al.*, 1988). The negative charge lends enough hydrophilicity to the crosslinker to allow direct addition of the reagent to aqueous reaction media without prior dissolution in organic solvents. Sulfo-MBS has the identical reactivity of MBS.

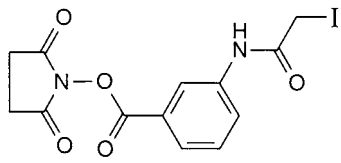
MBS was one of the first and most popular of the family of NHS ester–maleimide heterobifunctionals (Kitagawa and Aikawa, 1976). It has been used extensively to produce antibody–enzyme and other enzyme conjugates (Kitagawa *et al.*, 1978; O'Sullivan *et al.*, 1979; Freytag *et al.*, 1984a, b), in the preparation of hapten–carrier immunogens (Liu *et al.*, 1979; Lerner *et al.*, 1981; Kitagawa *et al.*, 1982; Niman *et al.*, 1985; Chamberlain *et al.*, 1989; Edwards *et al.*, 1989; Miller *et al.*, 1989; Swanson *et al.*, 1991), and for making immunotoxin conjugates (Youle and Nevelle, 1980; Dell'Arciprete *et al.*, 1988; Myers *et al.*, 1989). Additional applications include investigations of carnitine palmitoyltransferase-1 (Faye *et al.*, 2007), preparation of a targeting conjugate containing Cyt1Aa toxin directed at myeloma cells

(Cohen *et al.*, 2007), and an improved method for coupling synthetic peptide haptens to carrier proteins (Lateef *et al.*, 2007).

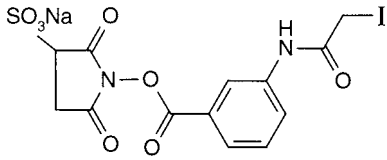
The generalized protocol for performing a multi-step conjugation reaction with MBS or sulfo-MBS is similar to that described for SMCC (Section 1.3, this chapter). Specific examples may be found in the cited references.

1.5. SIAB and Sulfo-SIAB

SIAB, *N*-succinimidyl(4-iodoacetyl)aminobenzoate, is a heterobifunctional crosslinker containing amine-reactive and sulfhydryl-reactive ends (Weltman, 1983). The NHS ester of SIAB can couple to primary amine-containing molecules, forming stable amide linkages (Chapter 2, Section 1.4). The other end contains an iodoacetyl group that is specific for coupling to sulfhydryl residues, creating stable thioether bonds (Chapter 2, Section 2.1). The aminobenzoate cross-bridge is a hydrophobic spacer that helps the reagent become fully permeable to membrane structures.



SIAB;
N-Succinimidyl(4-iodoacetyl)-
aminobenzoate
MW 402.15
10.6 Å



Sulfo-SIAB;
Sulfo-succinimidyl(4-iodoacetyl)-
aminobenzoate
MW 504.2
10.6 Å

Since SIAB is water-insoluble, it must be dissolved first in organic solvent prior to addition to an aqueous reaction medium. The most commonly used solvents for this purpose include DMSO and DMF. Typically, a concentrated stock solution is prepared in one of these solvents and an aliquot added to the protein conjugation solution. Long-term storage of the reagent in these solvents is not recommended, however, due to slow uptake of water and breakdown of the NHS ester end.

Conjugations done with SIAB usually proceed by a multi-step process. Because the crosslinker's NHS ester end is its most labile functionality, an amine-containing protein or molecule

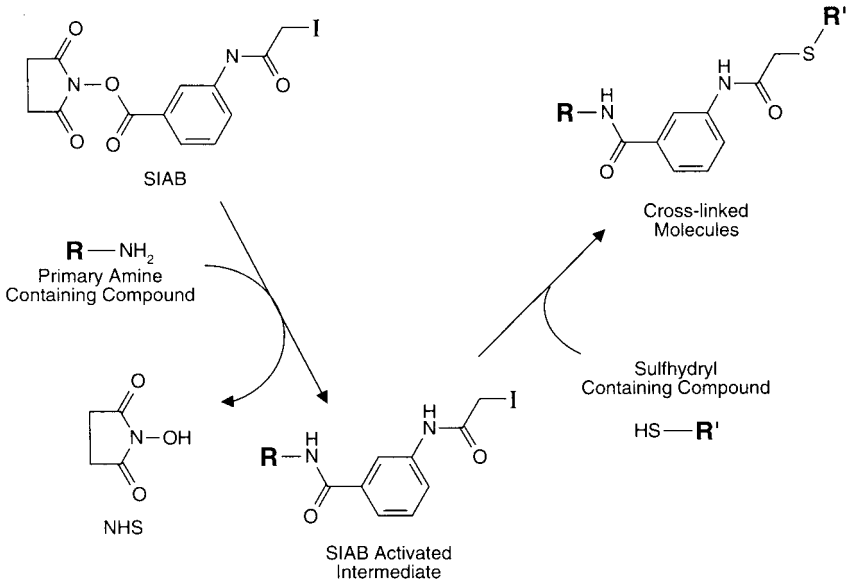


Figure 5.6 SIAB may be used to modify an amine-containing molecule for subsequent conjugation to a sulfhydryl-containing molecule.

is reacted first to create an iodoacetyl-activated intermediate (Figure 5.6). This iodoacetyl derivative is stable enough in aqueous solution to allow purification of the derivatized protein from excess reagent and other reaction by-products without significant loss of activity. The only consideration is to protect the iodoacetyl derivative from light, which may generate iodine and reduce the activity of the intermediate. Finally, the modified protein is mixed with a sulfhydryl-containing molecule to effect the conjugation through a thioether bond. The result of such two-step procedures is to direct the coupling toward only sulfhydryls on the second molecule while avoiding the polymerization problems that can occur with single-step protocols. Conjugations done with SIAB should avoid buffer components containing amines (i.e., Tris, glycine, or imidazole) or sulfhydryls (i.e., DTT, 2-mercaptoethanol, cysteine, etc.), since these will compete with the desired crosslinking reactions.

Sulfo-SIAB, sulfosuccinimidyl(4-iodoacetyl)aminobenzoate, is a water soluble analog of SIAB that contains a negatively charged sulfonate on its NHS ring. The negative charge lends enough hydrophilicity to the entire molecule to provide good solubility in aqueous solutions (up to about 10 mM). Sulfo-SIAB may be added directly to reaction mediums without prior dissolution in organic solvent, or solutions that are more concentrated may be made in water before transfer of an aliquot to the reaction to facilitate easy addition of small quantities. Aqueous stock solutions should be dissolved rapidly and used immediately to avoid excessive hydrolysis of the NHS ester.

SIAB and sulfo-SIAB have been used to make a high-capacity RNA affinity column for the purification of human IRP1 and IRP2 (Allerson *et al.*, 2003), to couple antibodies or Fab fragments to amine-modified microparticles (Härmä *et al.*, 2000), and in the attachment of oligonucleotides to surfaces for detection arrays (Adessi *et al.*, 2000).

The following protocol illustrates the use of SIAB in preparing antibody–enzyme conjugates using β-galactosidase.

Protocol

1. Dissolve a specific antibody to be conjugated at a concentration of 10 mg/ml in 50 mM sodium borate, 5 mM EDTA, pH 8.3 (reaction buffer).
2. Dissolve SIAB (Thermo Fisher) in DMSO at a concentration of 1.4 mg/ml. Alternatively, dissolve sulfo-SIAB in deionized water at a concentration of 1.7 mg/ml. Prepare fresh and use immediately. Protect from light.
3. Add 100 µl of the SIAB stock solution to each ml of the antibody solution. Mix gently to dissolve.
4. React for 1 hour at room temperature in the dark.
5. Purify the modified antibody by gel filtration on a desalting resin. Spin columns may be used to speed the separation process (Thermo Fisher). Perform the chromatography using the reaction buffer. To obtain good separation, apply sample at a ratio of no more than 8 percent of the total column gel volume. Monitor the eluting peak by using a small aliquot of each fraction and reacting it with a protein detection reagent such as Coomassie Protein Assay Reagent (Thermo Fisher) in a microplate. This avoids exposure of the entire modified protein fractions to UV light from a spectrophotometer, which could inactivate the iodoacetyl group. Collect the first peak eluting from the column, which contains the protein.
6. Add β-galactosidase to the activated antibody solution at a ratio of 4 mg of enzyme per mg of antibody.
7. React for 1 hour at room temperature in the dark.
8. To block any remaining iodoacetyl sites, add cysteine to a final concentration of 5 mM and react for an additional 15 minutes at room temperature.
9. Purify the conjugate by gel filtration using a buffer of choice (i.e., PBS, pH 7.4).

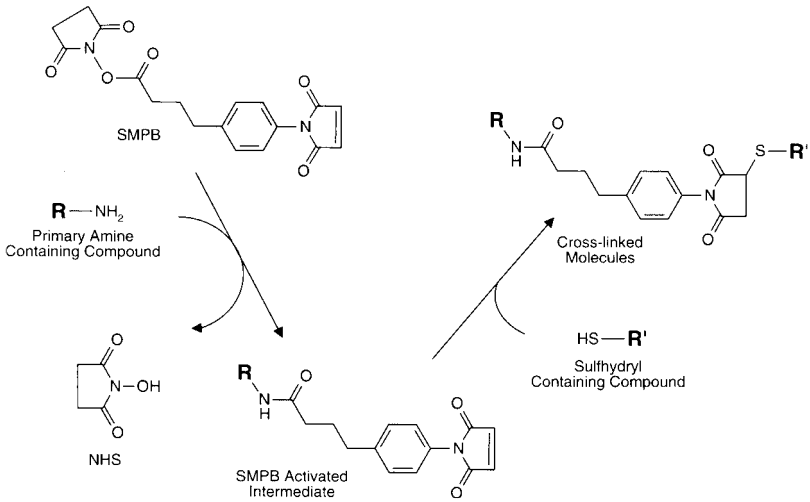
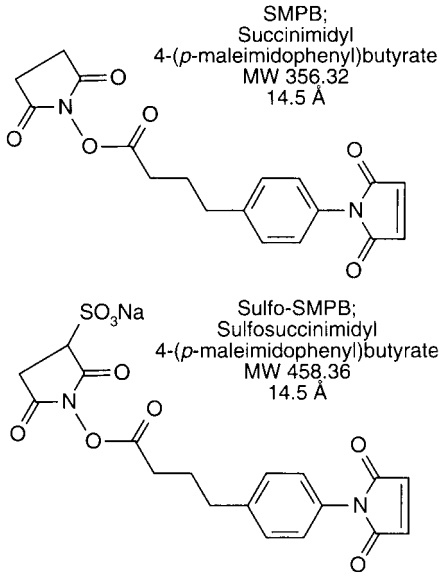


Figure 5.7 SMPB may be used in a two-step procedure to conjugate an amine-containing molecule to a sulfhydryl compound, forming amide and thioether bonds, respectively.

1.6. SMPB and Sulfo-SMPB

SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate, is a heterobifunctional analog of MBS (Section 1.4, this chapter) containing an extended cross-bridge (Thermo Fisher). The reagent has an amine-reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on the other end (Figure 5.7). Conjugates formed using SMPB thus are linked by stable amide and thioether bonds. A comparison with SPDP produced conjugates concluded that SMPB formed more stable complexes that survive *in vivo* for longer periods (Martin and Papahadjopoulos, 1982).



Conjugation reactions done with SMPB typically are multi-step procedures, wherein a protein is modified through its amine groups, purified to remove excess reagent, and then mixed with a sulfhydryl-containing molecule to effect the final conjugation. The maleimide group of SMPB is highly specific for coupling to sulfhydryl-containing proteins and other molecules, thus directing the conjugation to discrete points on the second molecule. This maleimide is, however, more labile to ring opening in aqueous solution than the maleimide group of SMCC due to its proximity to an aromatic ring. Therefore, the first protein modified with SMPB (to obtain a maleimide-activated intermediate) should be purified quickly to prevent extensive activity loss from hydrolysis and maleimide ring opening.

SMPB contains a hydrophobic cross-bridge and relatively nonpolar ends, which allows the reagent to permeate membrane structures. Due to its water-insolubility, it must be dissolved in an organic solvent prior to adding an aliquot to a reaction mixture. The solvents DMF and DMSO work well for this purpose. A concentrated stock solution prepared in these solvents allows for easy addition of a small amount to a conjugation reaction. Long-term storage in these solvents is not recommended due to slow water pickup and possible hydrolysis of the NHS ester end.

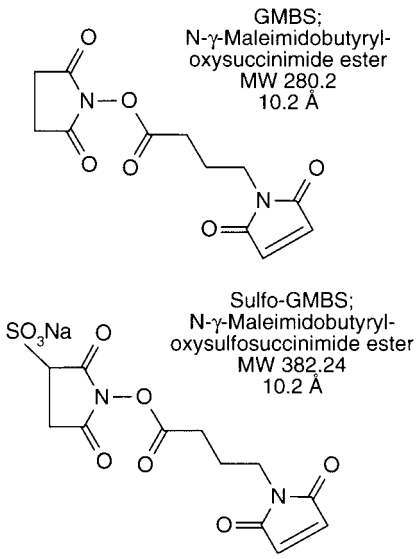
A water-soluble analog to SMPB, called sulfo-SMPB [sulfosuccinimidyl-4-(*p*-maleimidophenyl) butyrate] contains a negatively charged sulfonate group which lends considerable hydrophilicity to the molecule (Thermo Fisher). Sulfo-SMPB may be added directly to aqueous reaction

mixtures without prior dissolution in organic solvent. Concentrated stock solutions made in water should be dissolved quickly and used immediately to prevent hydrolysis of the NHS ester.

SMPB or sulfo-SMPB have been used to conjugate preformed vesicles and Fab′ fragments in a liposome carrier study (Martin and Papahadjopoulos, 1982), to attach insulin molecules to reconstituted Sendai virus envelopes (Gitman *et al.*, 1985a), for targeting of loaded virus envelopes by covalently attaching insulin molecules to receptor-depleted cells (Gitman *et al.*, 1985b), forming alkaline phosphatase–Fab′ fragment conjugates for an enzyme-linked immunosorbent assay (ELISA) (Teale and Kearney, 1986), preparing peptide–protein immunogen conjugates (Iwai *et al.*, 1988), studying the transport of the variant surface glycoprotein of Trypanosome brucia (Bangs *et al.*, 1986), and in preparing immunotoxin conjugates (Myers *et al.*, 1989). SMPB or sulfo-SMPB also have been used to modify glass slides for coupling thiol-modified oligonucleotides (Zhang *et al.*, 2006), for investigations of the protein decorin (Zhu *et al.*, 2005), and in creating conjugates with β-galactosidase that can cross the blood–brain barrier (Zhang and Pardridge, 2005).

1.7. GMBS and Sulfo-GMBS

GMBS, *N*-(γ-maleimidobutyryloxy)succinimide ester, is a heterobifunctional crosslinking agent that contains an NHS ester on one end and a maleimide group on the other (Fujiwara *et al.*, 1988) (Thermo Fisher). Its internal cross-bridge contains a linear 4-carbon spacer, resulting in 10.2 Å crosslinks between conjugated molecules (Figure 5.8). GMBS is water-insoluble and therefore must be dissolved in organic solvent. Typically, a concentrated stock solution is prepared in DMF or DMSO just before use, and then an aliquot of the solution is transferred to the aqueous reaction medium. The result is the formation of a micro-emulsion that effectively supplies crosslinker to the aqueous phase.



GMBS can be used in multi-step conjugation protocols wherein an amine-containing molecule or protein is first modified via the NHS ester end (its most labile-reactive group)to

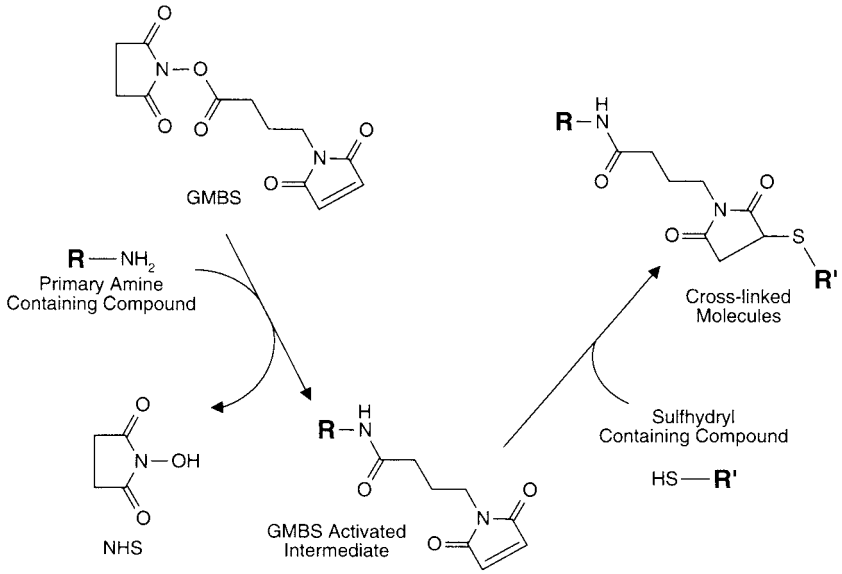


Figure 5.8 The reaction of GMBS with an amine-containing molecule yields a maleimide-activated intermediate that then can be used to crosslink with a sulfhydryl-containing compound.

create a stable amide bond. The derivative at this point contains reactive maleimidegroups able to couple with the available sulfhydryl groups on a second protein or molecule. This active intermediate then is purified to remove excess reagent and reaction by-products, and immediately added to the sulfhydryl-containing molecule to effect the final conjugation.

The maleimide group of GMBS is adjacent to an aliphatic spacer, so its stability toward ring opening should be better than crosslinkers like MBS which contain adjacent aromatic groups. Hydrolysis of the maleimide group results in loss of sulfhydryl coupling capability. However, GMBS is not as stable as the hindered maleimide group of SMCC, since the cyclohexane ring of that reagent inhibits hydrolysis and ring opening.

Sulfo-GMBS, *N*-(γ-maleimidobutyryloxy)sulfosuccinimide ester, is a water-soluble analog of GMBS containing a negatively charged sulfonate group on its NHS ring (Thermo Fisher). The charge provides enough hydrophilicity to allow at least 10 mM concentrations of the crosslinker to be made in aqueous reaction mediums. Its reactivity is identical to that of GMBS.

GMBS or sulfo-GMBS have been used for studying carnitine palmitoyltransferase-1 in its formation of a complex within the outer mitochondrial membrane (Faye *et al.*, 2007), for investigating protein organization of the postsynaptic density (Liu *et al.*, 2006), and in studying the structure and dynamics of rhodopsin (Jacobsen *et al.*, 2006).

The protocol for using GMBS or sulfo-GMBS in protein–protein crosslinking applications is similar to that of SMCC or sulfo-SMCC (see Section 1.3, this chapter).

1.8. SIAX and SIAXX

SIAX, succinimidyl-6-((iodoacetyl)amino)hexanoate, is a heterobifunctional reagent containing an NHS ester on one end and an iodoacetyl group on the other end (Brinkley, 1992)

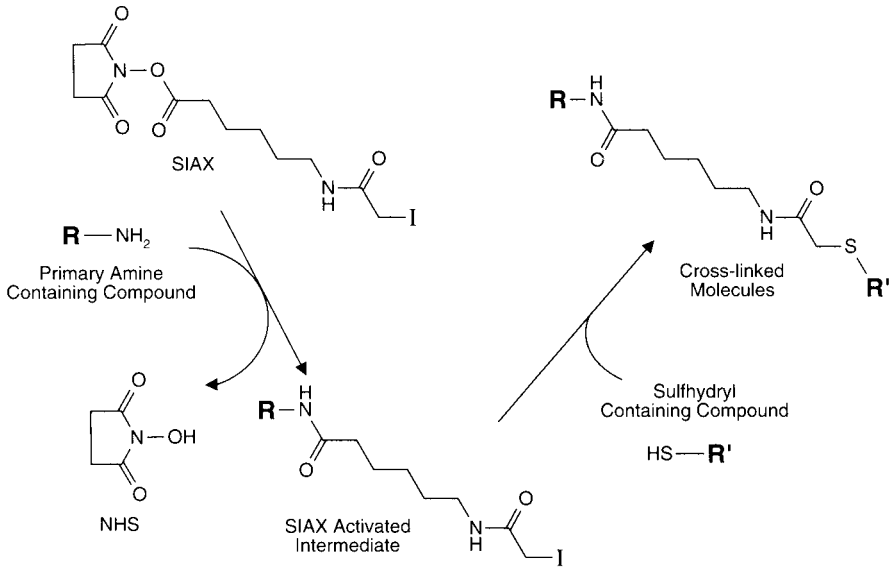
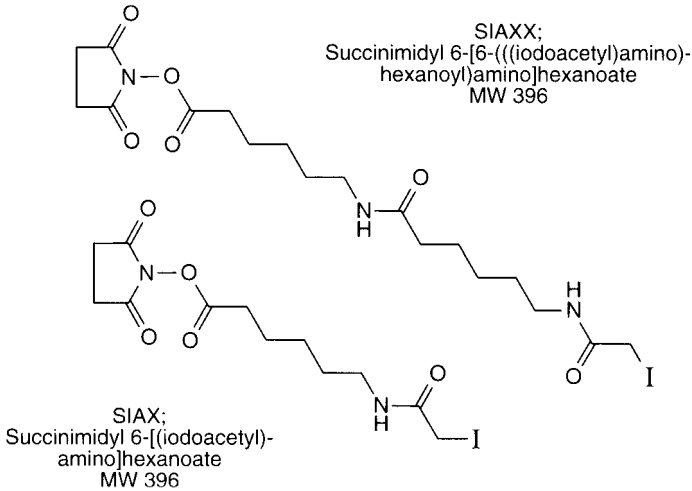


Figure 5.9 SIAX can be used to modify amine-containing molecules to produce sulfhydryl-reactive derivatives. Subsequent reaction with a thiol compound produces a thioether linkage.

(Invitrogen). The NHS ester reacts with primary amines in proteins and other molecules to form stable amide bonds. The iodoacetyl group is highly specific for sulfhydryl groups, reacting to create stable thioether linkages (Figure 5.9). The reactivity and use of this crosslinker is similar to SIAB, described previously. SIAX possesses a 6-aminohexanoic acid internal cross-bridge, providing a total of a 9-atom spacer between conjugated molecules.



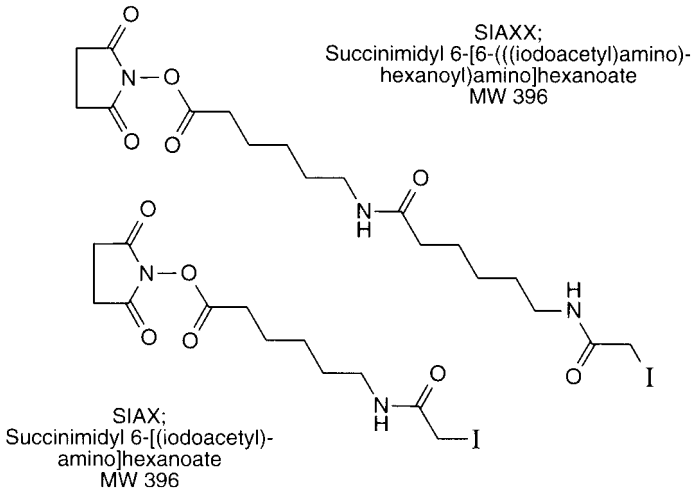
SIAX is a hydrophobic reagent that should penetrate membrane structures with good efficiency. The crosslinker must be solubilized in organic solvent (DMF or DMSO) prior to transferring a small amount to an aqueous reaction medium.

SIAXX, succinimidyl-6-(6-(((4-iodoacetyl)amino)hexanoyl)amino)hexanoate, is a long-chain analog of SIAX that contains two aminohexanoate spacer groups in its cross-bridge, instead of one (Invitrogen). Conjugates prepared with this reagent are connected by a spacer arm containing 16 atoms. Like SIAX, SIAXX must be first solubilized in DMF or DMSO before adding it to a buffered reaction. The increased chain length SIAXX, however, does not affect its reactivity toward amines and sulfhydryls.

Conjugation reactions done with SIAX or SIAXX are usually multi-step procedures similar to the protocol described for SIAB, previously.

1.9. SIAC and SIACX

SIAC, or succinimidyl-4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate, is a heterobifunctional reagent containing an NHS ester on one end and an iodoacetyl group on the other (Invitrogen). The crosslinker can react with amine groups via its NHS ester end to form stable amide bonds, while its iodoacetyl group can couple to sulfhydryls, creating stable thioether linkages (Figure 5.10). SIAC contains a cross-bridge made from a cyclohexane derivative, which provides approximately an 8-atom spacer between conjugated species.



SIAC is a hydrophobic crosslinker that must be solubilized in organic solvent (DMF or DMSO) prior to adding an aliquot to an aqueous reaction medium. It should exhibit good membrane permeability.

SIACX, or succinimidyl-6-((((4-(iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino) hexanoate, is an analog of SIAC that contains an additional aminohexanoate spacer group next to its NHS ester end (Invitrogen). The result is the creation of an approximately 16-atom spacer arm between conjugated molecules. All other properties of SIACX are similar to SIAC.

Conjugation reactions done with SIAC or SIACX are usually multi-step procedures similar to the protocol described for SIAB, previously.

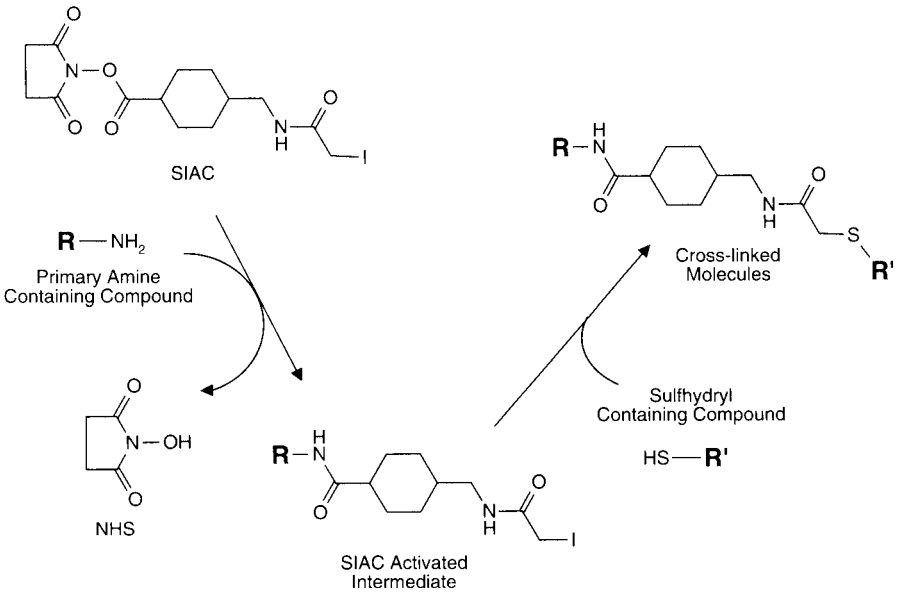
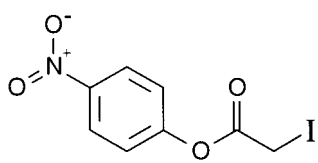


Figure 5.10 SIAC reacts with an amine-containing compound to yield an amide bond derivative that is reactive toward thiol-containing molecules. Secondary reaction with a sulfhydryl group gives a stable thioether bond.

1.10. NPIA

NPIA, or *p*-nitrophenyl iodoacetate, is a heterobifunctional reagent based upon an iodoacetyl group that has been activated at its carboxylic acid end with a *p*-nitrophenyl ester (Hudson and Weber, 1973; Huang *et al.*, 1975) (Invitrogen). This active ester species has much the same reactivity as an NHS ester, being highly reactive with amines at slightly basic pH values (pH 7–9). The *p*-nitrophenyl ester couples to amine-containing proteins and other molecules to form stable amide linkages. The other end of the short crosslinker can react with sulfhydryl groups to create thioether bonds. This is the smallest heterobifunctional iodoacetate-containing crosslinker available, forming only 2-atom cross-bridges between conjugated molecules (Figure 5.11). NPIA has been used to investigate close interactions between biological molecules (Hiratsuka, 1987; Sutoh and Hiratsuka, 1988).



NPIA
p-Nitrophenyl iodoacetate
MW 307

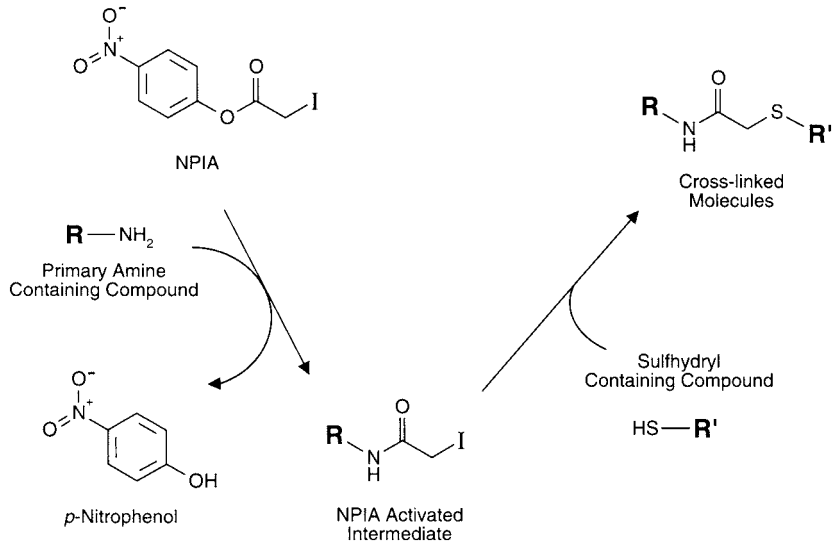


Figure 5.11 NPIA is one of the shortest heterobifunctional reagents. It reacts with amine-containing molecules through its *p*-nitrophenyl ester end to produce amide bonds. The iodoacetyl group then can be used to couple with thiol compounds to give stable thioether linkages.

NPIA is water-insoluble and should be dissolved in DMF or methylene chloride prior to addition of an aliquot to an aqueous reaction medium. Conjugation reactions done with NPIA are usually multi-step procedures similar to the protocol described for SIAB, previously.

2. Carbonyl-Reactive and Sulfhydryl-Reactive Crosslinkers

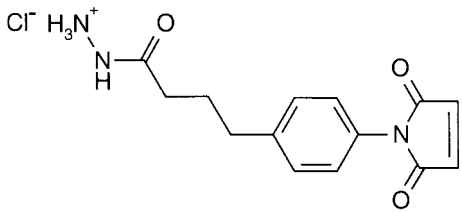
A relatively new set of heterobifunctional crosslinking agents now are available which contain a carbonyl-reactive group on one end and a sulfhydryl-reactive functionality on the other end. The main utility of these reagents is in conjugating carbohydrate-containing molecules, such as glycoproteins, to sulfhydryl-containing molecules. Both polysaccharide residues and sulfhydryl groups usually are present on proteins in limiting quantities and at discrete sites. In certain cases, conjugation through these groups can direct the coupling reaction away from critical active centers or binding sites, thus preserving activity of the proteins after crosslinking. A prime example of the advantages of this type of directed coupling can be seen when conjugating antibody molecules to other proteins, such as enzymes. The carbohydrate residues of immunoglobulin molecules often occur on the Fc portion, away from the antigen binding sites. Coupling procedures which direct the crosslinking reaction to parts on the antibody removed from the antigen binding sites have the best chance of retaining activity after conjugate formation. However, some antibodies do contain glycosylation sites in the Fab region of the molecule, thus making conjugation strategies through carbohydrates less certain as to their affect on antigen binding activity (Endo *et al.*, 1995; Mattu *et al.*, 1998).

The carbonyl-reactive group on these crosslinkers is a hydrazide that can form hydrazone bonds with aldehyde residues. To utilize this functional group with carbohydrate-containing molecules, the sugars first must be mildly oxidized to contain aldehyde groups by treatment with sodium periodate. Oxidation with this compound will cleave adjacent carbon–carbon bonds which possess hydroxyl groups, as are abundant in polysaccharide molecules (Chapter 1, Sections 2 and 4.4).

Two types of sulfhydryl-reactive functions are available on these reagents: pyridyl disulfide groups and maleimide groups. The pyridyl disulfide group will react with a sulfhydryl residue to create a disulfide bond. This linkage is reversible by treatment with a disulfide reducing agent. Reaction of a maleimide group with a sulfhydryl, however, forms a permanent thioether bond of good stability. Thus, either reversible or permanent conjugates may be designed using these heterobifunctionals.

2.1. MPBH

MPBH, or 4-(4-*N*-maleimidophenyl)butyric acid hydrazide, is a heterobifunctional crosslinking agent that contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide on the other end (Thermo Fisher). The cross-bridge between the two functional ends provides a 17.9 Å spacer. The hydrazide group is produced as the hydrochloride salt. The reagent as a whole has good water solubility. It can be dissolved in 0.1 M sodium acetate, pH 5.5, up to a concentration of 327 mg/ml. It is also freely soluble in DMSO and may be stored as a concentrated stock solution in this solvent without degradation.



MPBH
4-(4-N_Maleimidophenyl)butyric
acid hydrazide hydrochloride
MW 309.75
17.9 Å

The maleimide group of MPBH is adjacent to an aromatic ring and thus may exhibit instability to hydrolysis in aqueous solutions, especially at alkaline pH. Hydrolysis opens the maleimide ring and destroys its coupling ability with sulfhydryls. However, both reactive ends of the crosslinker are stable enough to survive a multi-step coupling protocol without extensive loss of activity. Thus, a sulfhydryl-containing protein or molecule may be modified via the maleimide end of MPBH, the derivative purified by gel filtration to remove excess reactants, and then mixed with a glycoprotein (that has been previously oxidized to provide aldehyde residues) to effect the final conjugation (Figure 5.12). The opposite approach also is possible: modification of the glycoprotein first, purification, and subsequent mixing with a sulfhydryl-containing molecule. With this second option, however, the purification step should be done quickly to prevent extensive hydrolysis of the maleimide group.

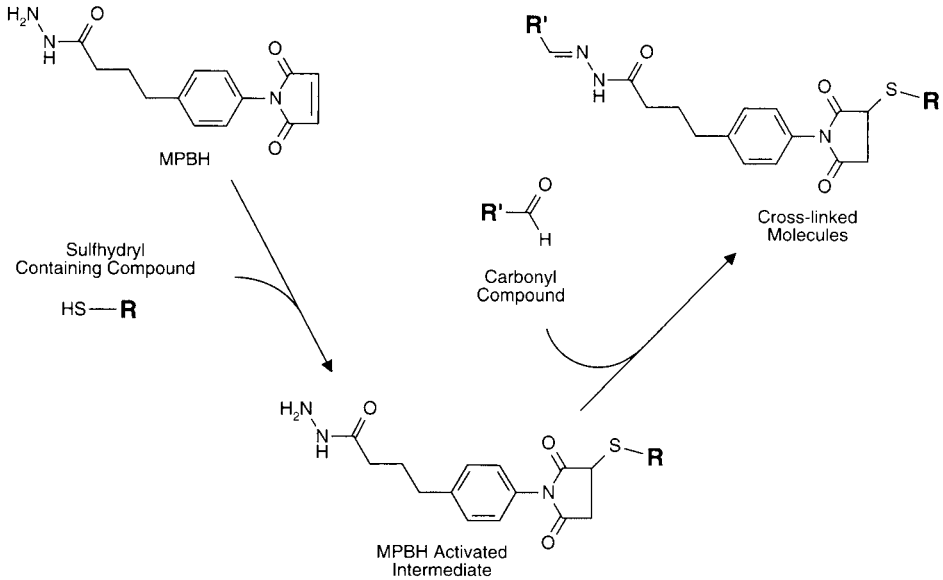
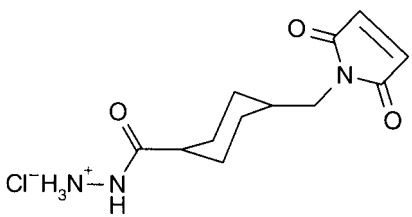


Figure 5.12 MPBH reacts with sulfhydryl-containing molecules through its maleimide end to produce thioether linkages. Its hydrazide group then can be used to conjugate with carbonyl-containing molecules (such as periodate-oxidized carbohydrates that contain aldehydes) to give hydrazone bonds.

MPBH has been used to conjugate CD4 without loss of biological activity (Chamow *et al.*, 1992).

2.2. M_2C_2H

M_2C_2H, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide, is a heterobifunctional crosslinking agent that contains a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive maleimide group on the other end (Thermo Fisher). The reagent is similar to MPBH (described previously), but the maleimide group on M_2C_2H is expected to be more stable in aqueous solutions, since it is adjacent to an aliphatic cyclohexane ring instead of an aromatic phenyl group. In this sense, the cross-bridge of M_2C_2H is nearly identical to that of SMCC, which contains one of the most stable maleimide groups known. The hydrophobic, hindered environment



M_2C_2H
4-(N-Maleimidomethyl)cyclohexane-
1-carboxyl-hydrazide hydrochloride
MW 287.75
15.1 Å

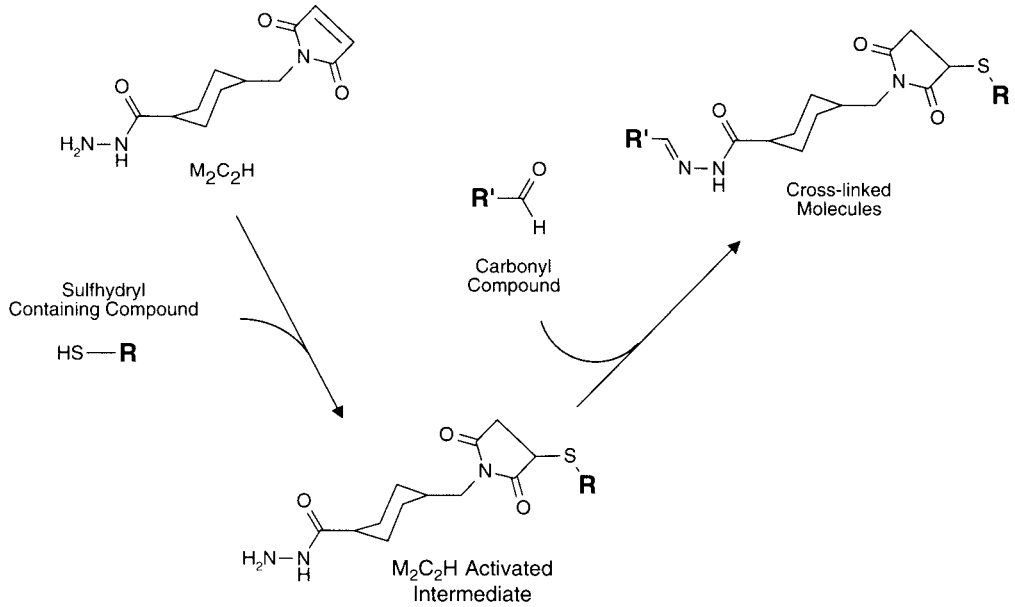


Figure 5.13 M_2C_2H can be used to crosslink a sulfhydryl-containing molecule with an aldehyde-containing compound. Glycoproteins may be conjugated using this reagent after treatment with sodium periodate to form reactive aldehyde groups.

of the cyclohexane ring should provide similar stability advantages to this reagent. Reaction of the maleimide group with a sulfhydryl residue results in the formation of a stable thioether bond.

On the other end of the crosslinker, the hydrazide functional group can react with periodate-oxidized carbohydrate molecules or with the reducing end of carbohydrates to form hydrazone linkages (Chapter 1, Sections 2 and 4.5). Thus, glycoproteins can be targeted specifically at their polysaccharide chains, avoiding crosslinking at active sites, which can lead to activity losses (Figure 5.13).

M_2C_2H is slightly soluble in aqueous solutions, reportedly having a maximal solubility of 3.2 mg/ml in 0.1 M sodium acetate at pH 5.5. It is also soluble in organic solvents, which allows for the preparation of concentrated stock solutions to be made prior to addition of a small aliquot to an aqueous reaction mixture. The crosslinker is particularly stable in acetonitrile.

2.3. PDPH

PDPH, 3-(2-pyridyldithio)propionyl hydrazide, is a heterobifunctional reagent that possesses a carbonyl-reactive hydrazide group on one end and a sulfhydryl-reactive pyridyl disulfide group on the other end (Thermo Fisher). Thus, sulfhydryl-containing proteins or other thiol molecules may be conjugated to carbohydrate-containing molecules (after treatment of the polysaccharide portion with sodium periodate to create aldehyde residues) (Figure 5.14). Using this crosslinker, glycoproteins can be coupled specifically through their carbohydrate chains, in many cases better avoiding active centers or binding sites than when coupling through abundant polypeptide

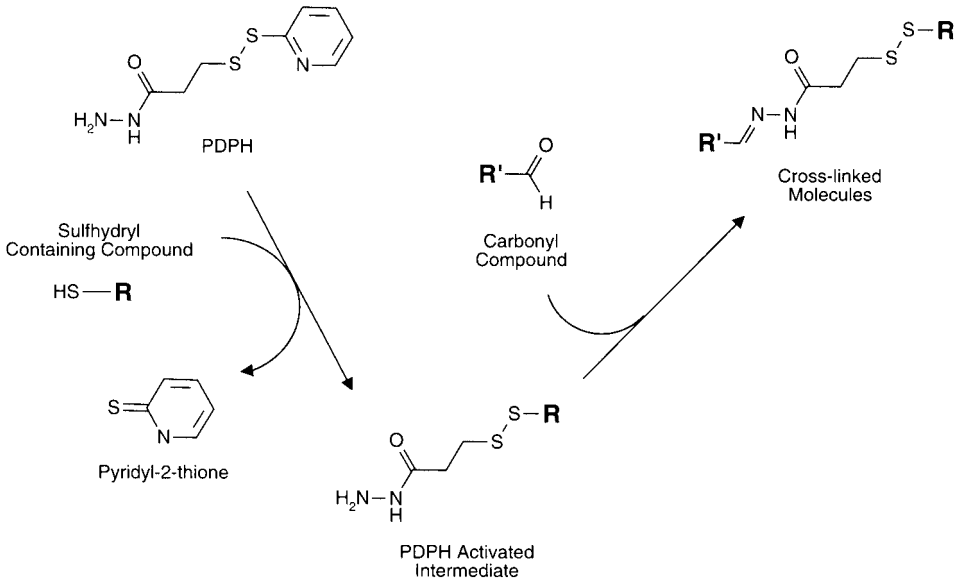
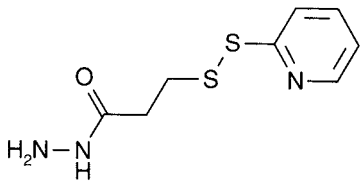


Figure 5.14 PDPH reacts with thiol-containing compounds through its pyridyl disulfide end to form reversible disulfide linkages. Its hydrazide end then may be subsequently conjugated with an aldehyde-containing molecule to form hydrazone bonds. Glycoproteins may be crosslinked using this approach after periodate activation to generate aldehyde groups.

groups like amines. Since the pyridyl disulfide group reacts with sulfhydryls to create disulfide bonds, the crosslinked proteins can be cleaved by reduction with DTT (Chapter 1, Section 4.1).



PDPH also may be used as a thiolation reagent to add sulfhydryl functional groups to carbohydrate molecules. The reagent can be used in this sense similar to the protocol described for AMBH (Chapter 1, Section 4.1). After modification of an oxidized polysaccharide with the hydrazide end of PDPH, the pyridyl group is removed by treatment with DTT, leaving the exposed sulfhydryl (Figure 5.15).

PDPH is soluble in 0.1 M sodium acetate, pH 5.5, at a maximal concentration of 14.2 mg/ml. The reagent is particularly stable in acetonitrile for preparation of concentrated stock solutions.

PDPH has been used in the preparation of immunotoxin conjugates (Zara *et al.*, 1991). It has also been used to create a unique conjugate of nerve growth factor (NGF) with an

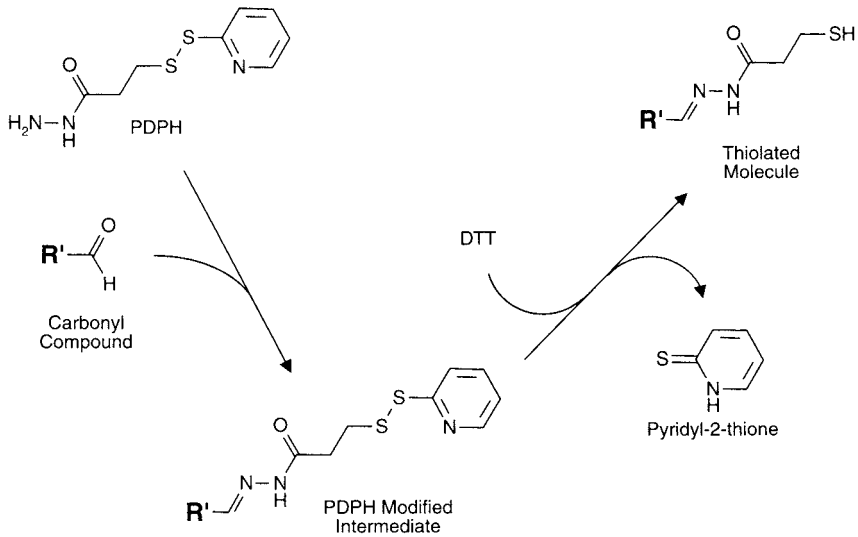


Figure 5.15 PDPH may be used to add a sulfhydryl group to an aldehyde-containing molecule. After reacting its hydrazide end with the aldehyde to form a hydrazone bond, the pyridyl disulfide may be reduced with DTT to create a free thiol.

antibody directed against the transferrin receptor OX-26, which could traverse the blood–brain barrier (Friden, 1993). Labeling of antibody molecules with PDPH at oxidized polysaccharide sites followed by reduction to free the sulfhydryl has been used to form a technetium-99 m complex for radiopharmaceutical use (Ranadive *et al.*, 1993) (Chapter 10, Section 5). PDPH also has been used to study the uptake of rsCD4 across the blood–brain barrier (Walus *et al.*, 1996), to prepare an NGF conjugate (Bäckman *et al.*, 1996), and to study novel engineered cell-surface receptors (Lee *et al.*, 1999).

3. Amine-Reactive and Photoreactive Crosslinkers

An important class of heterobifunctional reagents is the photoreactive crosslinkers that have one end that can be photolyzed to initiate coupling. Photoreactive crosslinkers may be designed to utilize any one of a number of photosensitive groups, including aryl azides, fluorinated aryl azides, benzophenones, anthraquinones, certain diazo compounds, and diazirine derivatives (Chapter 2, Section 7). The best photoreactive groups are stable in aqueous solution in the dark, and may be activated at the desired time by a pulse of light at the appropriate wavelength. The other end of these heterobifunctionals usually contains a spontaneously reactive functionality that will couple rapidly with certain groups present on target molecules. This secondary functionality is sometimes called *thermoreactive* to differentiate it from the photoreactive end and to emphasize its ready-reactivity or sometimes its labile nature in aqueous environments. The thermoreactive end is typically amine-reactive, sulfhydryl-reactive, carbonyl-reactive, carboxylate-reactive, or arginine-reactive. Still another class of photoreactive heterobifunctionals may use a

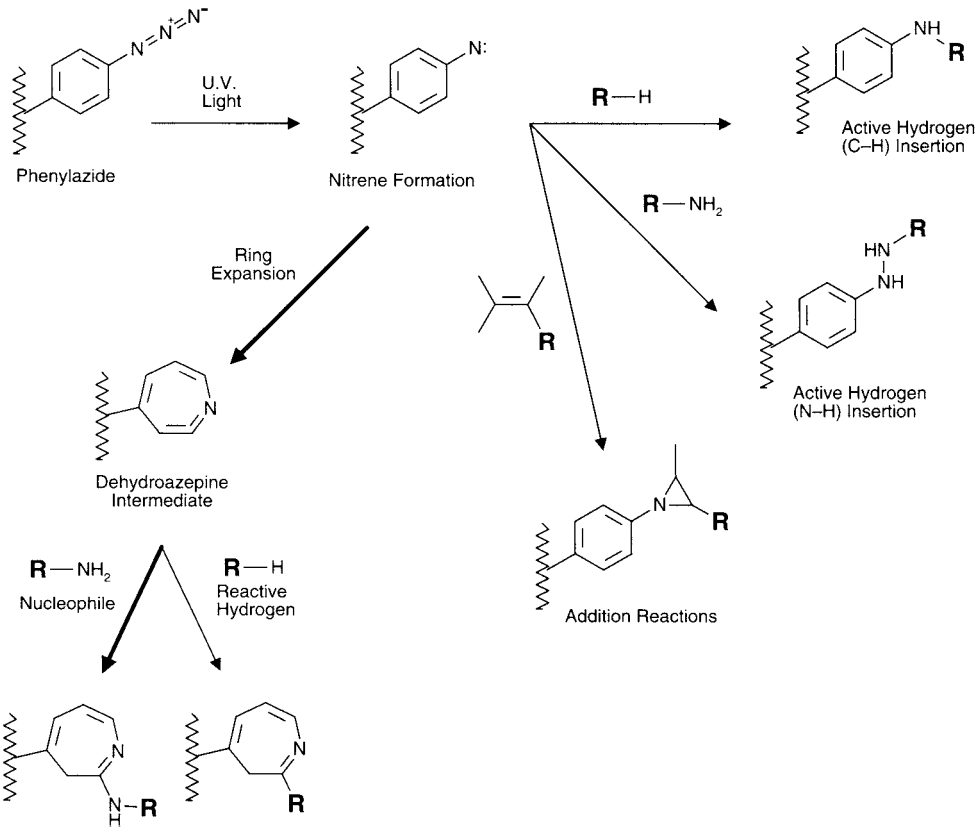


Figure 5.16 Photoactivation of a phenyl azide group with UV light results in the formation of a short-lived nitrene. Nitrenes may undergo a number of reactions, including insertion into active carbon–hydrogen or nitrogen–hydrogen bonds and addition to points of unsaturation in carbon chains. The most likely route of reaction, however, is to ring-expand to a dehydroazepine intermediate. This group is highly reactive toward nucleophiles, especially amines.

biotin handle at one end to crosslink specifically, but noncovalently, with avidin or streptavidin molecules (Chapter 11, Section 4).

Photoreactive groups can be categorized by the reactive species that is generated upon photolysis. The most popular type of photosensitive group, an aryl azide derivative, forms a short-lived nitrene that reacts extremely rapidly with the surrounding chemical environment (Gilchrist and Rees, 1969). Recent evidence, however, indicates that the photolyzed intermediates of aryl azides can undergo ring-expansion to create nucleophile-reactive dehydroazepines. Instead of inserting nonselectively at active carbon–hydrogen bonds, dehydroazepines have a tendency to react preferentially with nucleophiles, especially amines (Figure 5.16). However, some investigators have shown that aryl azides that possess a perfluorinated ring structure or are substituted completely with halogen atoms are quite efficient at forming the desired nitrene intermediate (Keana and Cai, 1990; Cai *et al.*, 1993; Schnapp and Platz, 1993; Schnapp *et al.*, 1993; Soundararajan *et al.*, 1993; Yan *et al.*, 1994). A few crosslinking reagents now use

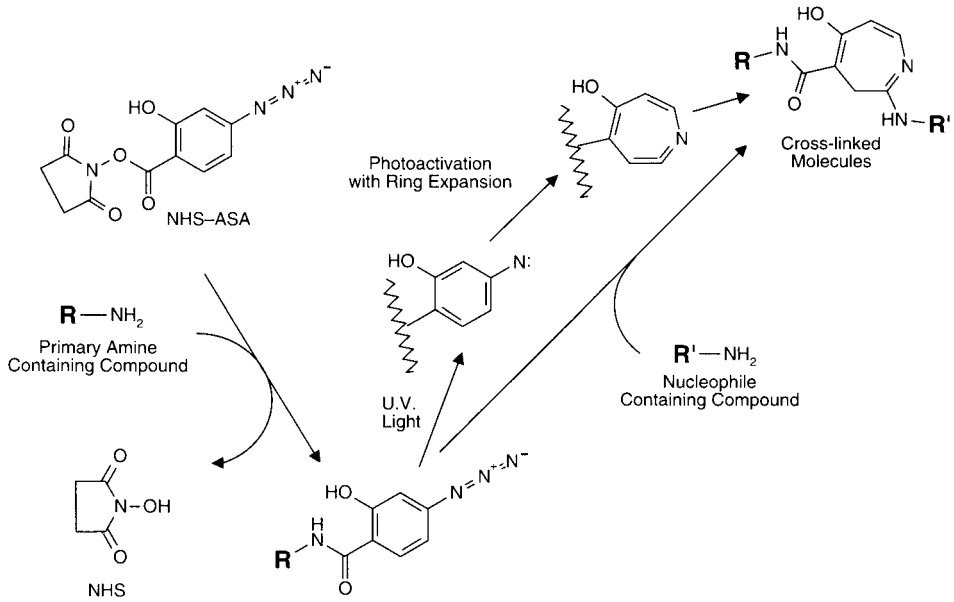


Figure 5.17 NHS-ASA reacts with amine-containing compounds to form stable amide linkages. Photoactivation with UV light results in ring expansion to a dehydroazepine intermediate, which can react with amines to form covalent bonds.

halogen-substituted phenyl azides to provide greater efficiency of photoreactive insertion into target molecules (Chapter 28, Sections 2.2 and 3.2).

One advantage of aryl azide photoreactive crosslinkers is that they have a relatively low energy of activation, which is optimal in the long UV region. In addition, many aryl azides possess nitro groups on their associated aromatic ring structures. These electron-withdrawing groups tend to increase the optimal wavelength for photolysis upwards close to the 350 nm range. The benefit of this approach is that relatively low light exposure at the higher-energy UV wavelengths avoids potential bond breakage that may occur with some sensitive compounds upon photolysis. In addition, some biological molecules can undergo crosslinking reactions upon irradiation with UV light of less than 300 nm (e.g., DNA).

Other phenyl azide-containing reagents possess hydroxyl groups on their aromatic rings. These electron-donating groups activate the ring system to allow electrophilic substitution reactions to occur on the crosslinker prior to its use. A major application of this ability is to radioiodinate the photoreactive end, thus permitting crosslinking and detection of proteins within samples.

Suitable light sources for photolyzing include sunlamps manufactured by a number of companies, such as Philips Ultrapnil MLU 300 W, General Electric sunlamp RSM 275 W, or National Self-Ballasted BHRF 240–250 V 250 W W-P lamp. Irradiation for 15 minutes with such lamps while the sample is cooled in an ice bath will result in good photolysis of photoreactive crosslinkers and modification reagents. In addition, many long-wavelength UV light sources with irradiation capability at about 366 nm work well.

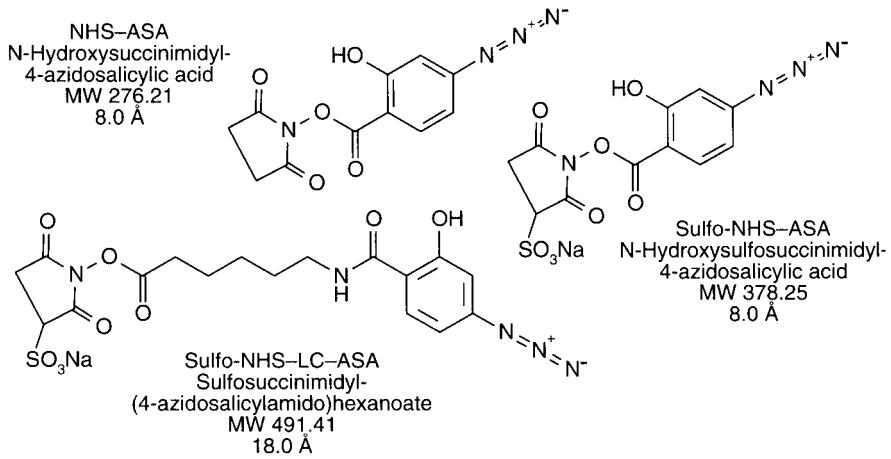
Although photoreactive aryl azides are relatively inert to thermochemical reactions prior to photolysis, they are not stable in the presence of sulfhydryl compounds which can reduce the

azide functionality to an amine with concomitant release of N_2. Avoid, therefore, the use of reductants such as DTT or 2-mercaptoethanol before the photoreaction step, as these can react with the aryl azide within minutes (Staros *et al.*, 1978). Also, avoid amine-containing buffer components such as Tris or glycine, because of the potential for nucleophilic reactivity with the photolyzed dehydroazepine intermediate formed from photolysis of unsubstituted phenyl azides.

Of the following amine-reactive and photoreactive crosslinkers, the overwhelming majority use an aryl azide group as the photosensitive functional group. Only a few use alternative photoreactive chemistries, particularly perfluorinated aryl azide, benzophenone, or diazo compounds. For general background information on photoreactive crosslinkers, see Das and Fox (1979), Kiehm and Ji (1977), Vanin and Ji (1981), and Brunner (1993).

3.1. NHS-ASA, Sulfo-NHS-ASA, and Sulfo-NHS-LC-ASA

NHS-ASA (*N*-hydroxysuccinimidyl-4-azidosalicylic acid) is a heterobifunctional reagent containing an NHS ester on one end and a photoreactive aryl azide group on the other (Thermo Fisher). The amine-reactive NHS ester can be reacted with proteins or other primary amine-containing molecules to yield a photosensitive derivative suitable for probing biological interaction sites. Upon photolysis with a long UV light source, the aryl azide end is activated to covalently complex with closely associated target molecules (Figure 5.17). The small cross-bridge of NHS-ASA is built from a salicylate derivative that contains a hydroxyl group on the aromatic ring. The ring-activating nature of this group provides an iodination site on the crosslinker to allow tracking of modified molecules (Ji and Ji, 1982) (Chapter 12, Section 5).

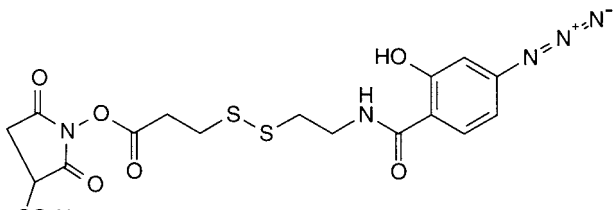


Reported applications of NHS-ASA include photoaffinity labeling of ^{125}I-ASA-Con A to erythrocyte ghosts (Ji and Ji, 1982), derivatization of human choriogonadotropin with ^{125}I-NHS-ASA with photo-initiated crosslinking of the α–β dimer (Ji *et al.*, 1985), radiolabeling of D-glucose and conjugation of the sugar to the human erythrocyte monosaccharide transporter protein (Shanahan *et al.*, 1985), photoaffinity labeling of a bacterial sialidase (van der Horst *et al.*, 1990), and identification of the peptide binding site of DnaK (Zhang and Walker, 1996).

Two analogs of NHS-ASA that provide alternative physical characteristics are available. Sulfo-NHS-ASA is a water-soluble version of the crosslinker that contains a negatively charged sulfonate group on its NHS ring. Sulfo-NHS-LC-ASA also has the water solubility advantage provided by a sulfonate, but it possesses a longer cross-bridge made from a 6-aminocaproic acid chain in its internal structure. The longer spacer increases the potential distance between conjugated molecules, thus allowing more flexibility in the experimental design. Both analogs are still iodinatable to provide radiolabeling capability.

3.2. SASD

SASD (sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate) is a heterobifunctional crosslinker containing a photoreactive group and an amine-reactive NHS ester (Thermo Fisher). The NHS ring possesses a negatively charged sulfonate group which lends water solubility to the reagent. The cross-bridge of SASD contains a central disulfide group that provides cleavability after conjugation. Reaction with a disulfide reducing agent such as DTT breaks the disulfide bond and releases the crosslinked molecules. The photosensitive end of SASD is built from a salicylic acid derivative which contains a ring-activating hydroxyl group. Due to the presence of this group, the crosslinker can be radiolabeled with ^{125}I prior to a conjugation reaction. Iodination occurs *ortho* or *para* to the hydroxyl group on the phenyl ring, next to the aryl azide function (Figure 5.18) (Chapter 12, Section 5).



SASD
Sulfosuccinimidyl-2-(*p*-azido-salicylamido)ethyl-1,3'-dithiopropionate
MW 541.51
18.9 Å

The combination of radiolabeling and cleavability provides the ability to detect the fate of the protein that retains the radiolabel after disulfide reduction. Thus, for investigations involving biomolecular interactions, a purified protein can be labeled with SASD through its amine groups via the NHS ester end of the crosslinker, allowed to interact *in vivo* with unknown target proteins, and photolyzed to effect a crosslink with these unknown substances. Subsequently the complex can be localized in the cell or effectively isolated by following the radiolabel. Alternatively, the conjugate can be cleaved by reduction, which results in the label being transferred to the unknown interacting protein, and its fate determined or the identity of the unknown protein revealed through the radiolabel (Figure 5.19). Such label transfer reagents are described in more detail in Chapter 28.

Reported applications of SASD involve modification of lipopolysaccharide (LPS) molecules and studying their interaction with albumin and an antibody directed against LPS (Wollenweber and Morrison, 1985), identification of the murine interleukin-3 receptor and an *N*-formyl peptide receptor (Sorenson *et al.*, 1986), crosslinking of factor V and Va to iodinated peptides

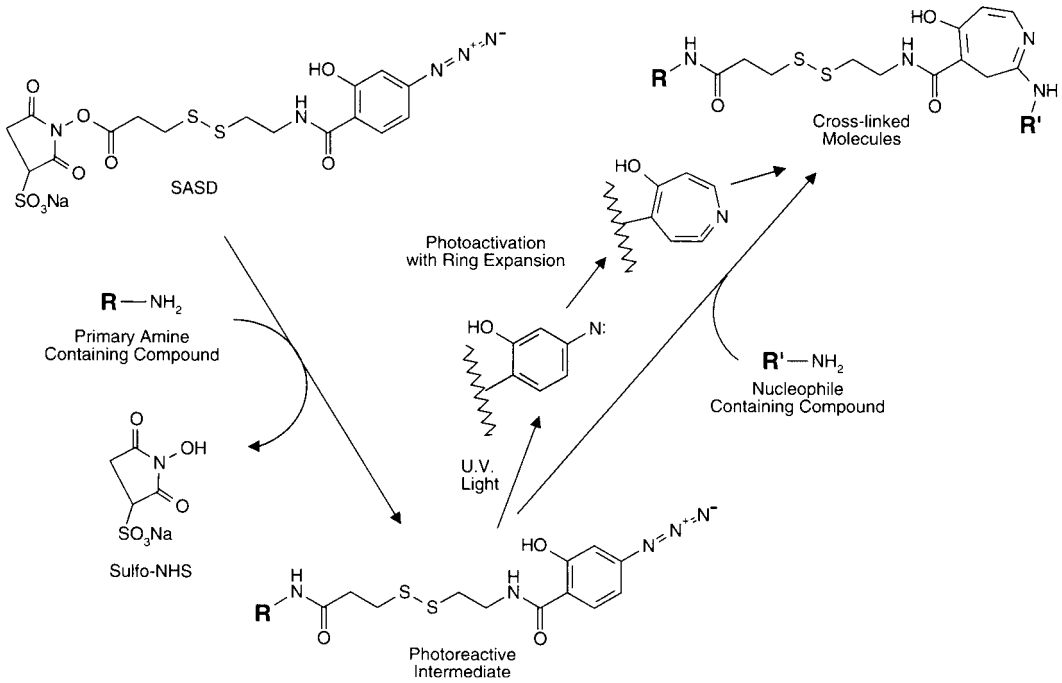


Figure 5.18 SASD is a photoreactive crosslinker that can be used to modify amine-containing compounds through its NHS ester end and subsequently photoactivated to initiate coupling with nucleophiles (after ring expansion to an intermediate dehydroazepine derivative). The crosslinks may be selectively cleaved at the internal disulfide group using DTT.

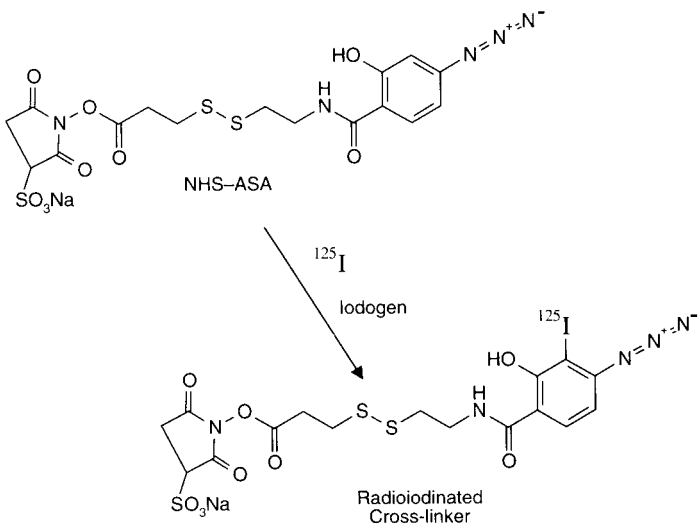


Figure 5.19 The hydroxyl group on the phenyl azide ring of SASD may be iodinated with ^{125}I to allow radiolabeling studies to be done on photolyzed conjugates.

(Chattopadhyay *et al.*, 1992), and a comparison of radiolabeling techniques for the crosslinker (Shephard *et al.*, 1988). Other studies have involved the investigation of protein interactions using the label transfer nature of radioiodinated SASD (Gupta *et al.*, 2005; Lindersson *et al.*, 2005; LeFebvre *et al.*, 2006).

The best radiolabeling technique for SASD is to use the Iodogen method (Shephard *et al.*, 1988) described in Chapter 12, Section 3. The following suggested protocol for using SASD was based on the method described in the Thermo Fisher Catalog.

Protocol

The following operations should be done using standard safety procedures for working with radioactive compounds. All steps involving SASD prior to initiation of the photoreaction should be done protected from light to avoid loss of phenyl azide activity. The radiolabeling procedure should be done quickly to prevent excessive loss of NHS ester activity due to hydrolysis.

1. Radiolabel 55 nmol of SASD using IODO-GEN (Thermo Fisher) and 40 μCi Na ^{125}I for 30 seconds. Do not use chloramine-T, since termination of the iodination reaction with this reagent involves addition of a reducing agent which may cleave the disulfide bonds of the crosslinker.
2. Terminate the iodination by removing the SASD solution from the IODO-GEN reagent using a transfer pipette. Be careful not to carry any solid IODO-GEN reagent with the transfer. Since free radioactive iodine still may be present in the solution, it may be necessary to add an iodine scavenger to prevent the possibility of radiolabels being incorporated into the proteins being crosslinked. Suitable scavengers include tyrosine or *p*-hydroxyphenylacetic acid. Adding these compounds in molar excess to the amount of iodine present will prevent any secondary modifications from occurring. Immediately add the radiolabeled SASD solution to the equivalent of 16 nmol of a protein to be modified. The protein should be dissolved previously in a minimum quantity of 0.1 M sodium borate, pH 8.4 (conjugation buffer). The more concentrated the protein, the more efficient will be the modification reaction.
3. React for 30 minutes to create the SASD derivative, coupled through the NHS ester-reactive group of the crosslinker onto available amine groups of the protein (forming amide bonds).
4. Purify the modified protein by desalting using a desalting resin and performing the chromatography using a buffer of choice. Pool fractions containing protein. The protein should be radiolabeled at this point and also contain photoreactive phenyl azide groups from the SASD modification.
5. Add the SASD-modified protein to a second protein or other molecule to be conjugated. After mixing, expose the solution to long-wave UV light for 10–15 minutes at room temperature to effect the conjugation. The solution may be kept on ice to prevent over-heating of sensitive proteins.

3.3. HSAB and Sulfo-HSAB

HSAB (*N*-hydroxysuccinimidyl-4-azidobenzoate) is a heterobifunctional reagent containing an amine-reactive NHS ester on one end and a photoreactive phenyl azide group on the other end

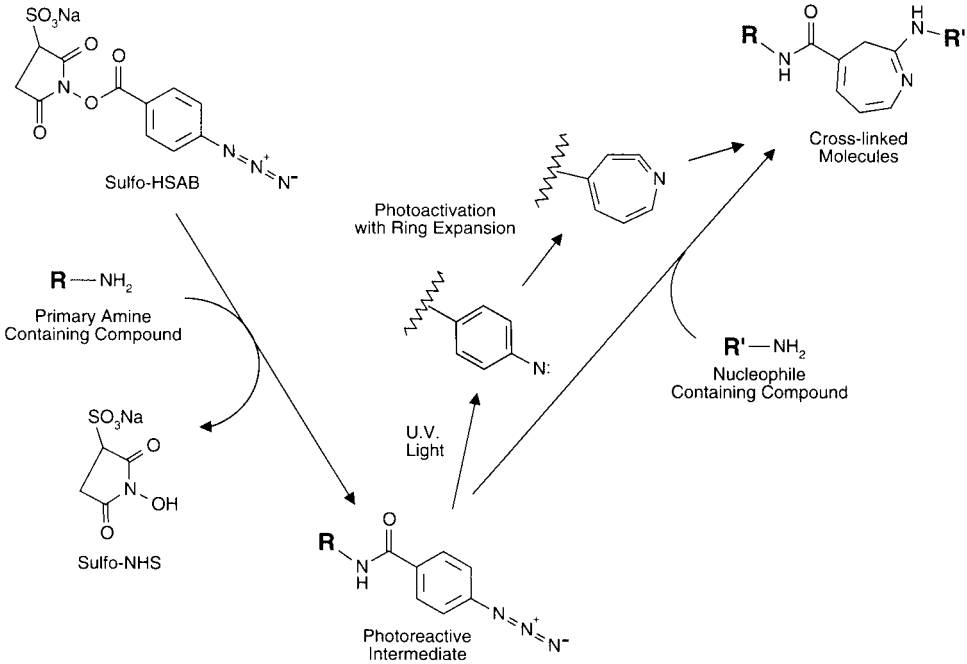
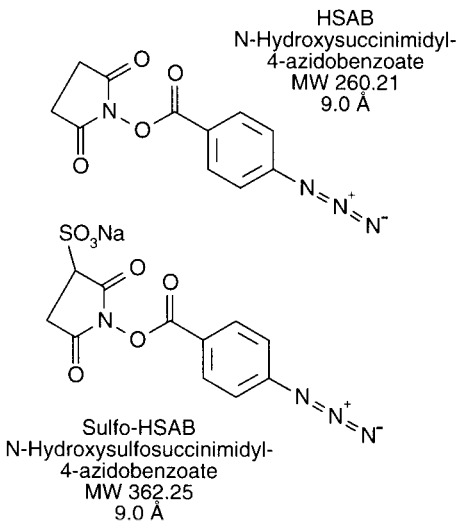


Figure 5.20 Sulfo-HSAB is a short photoreactive crosslinker that can be used to modify amine-containing molecules through its NHS ester end to form amide linkages. After photoactivation, the phenyl azide group can react with amines to create a covalent bond.

(Thermo Fisher). The small cross-bridge, built from a benzoic acid group, provides crosslinking ability at short intermolecular distances. Reaction of one protein via the NHS ester end of the crosslinker provides a stable derivative that can be incubated with a target molecule and then photolyzed to effect the final conjugation (Figure 5.20).



Reactions done with HSAB should involve dissolution of the crosslinker in organic solvent prior to addition to an aqueous reaction medium. DMSO or DMF are suitable solvents to prepare concentrated stock solutions. Protect all solutions from light to avoid loss of photoreactive phenyl azide groups prior to the desired point of photolysis.

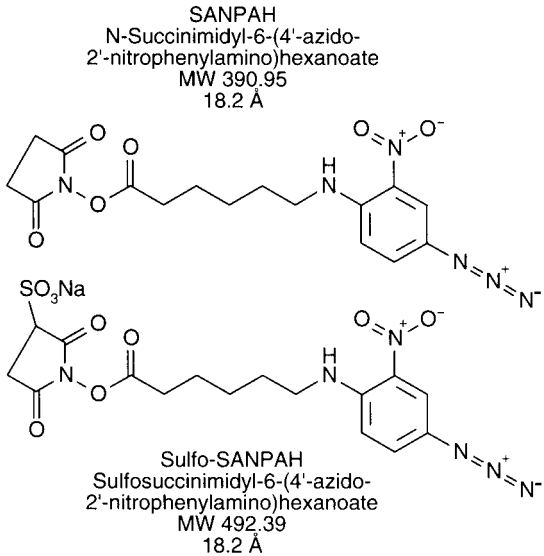
Reported applications of HSAB include photoaffinity labeling of peptide hormone binding sites (Galardy *et al.*, 1974), photoaffinity labeling of the insulin receptor with derivatized insulin analog (Yeung *et al.*, 1980), identifying NGF receptor proteins in sympathetic ganglia membranes (Massague *et al.*, 1981), labeling of the hormone receptor of both α and β subunits of human choriogonadotropin (Ji and Ji, 1981), isolation of *in situ* crosslinked ligand–receptor complexes (Ballmer-Hofer *et al.*, 1982), and crosslinking vasoactive intestinal polypeptide to its receptors on intact human lymphocytes (Wood and O'Dorisio, 1985).

Sulfo-HSAB, *N*-hydroxysulfosuccinimidyl-4-azidobenzoate, is a water soluble analog of HSAB possessing a negatively charged sulfonate group on its NHS ring. This crosslinker may be added directly to aqueous reaction media without prior dissolution in organic solvent. To aid in the addition of small quantities of the reagent, a concentrated solution of sulfo-HSAB may be made in water and then an aliquot added to the reaction. Aqueous stock solutions should be dissolved quickly and used immediately to prevent extensive hydrolysis of the NHS ester.

HSAB and sulfo-HSAB have been used to investigate serum amyloid A (Cai *et al.*, 2007), the functional role of C-terminal sequence elements in the transporter associated with antigen processing (Ehses *et al.*, 2005), and the kinetics of intermolecular interactions during cytochrome C protein folding (Nishida *et al.*, 2004).

3.4. SANPAH and Sulfo-SANPAH

SANPAH (*N*-succinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate) is a heterobifunctional crosslinking agent containing an NHS ester and a photoreactive phenyl azide group (Thermo Fisher). The NHS ester end can react with amine groups in proteins and other molecules, forming



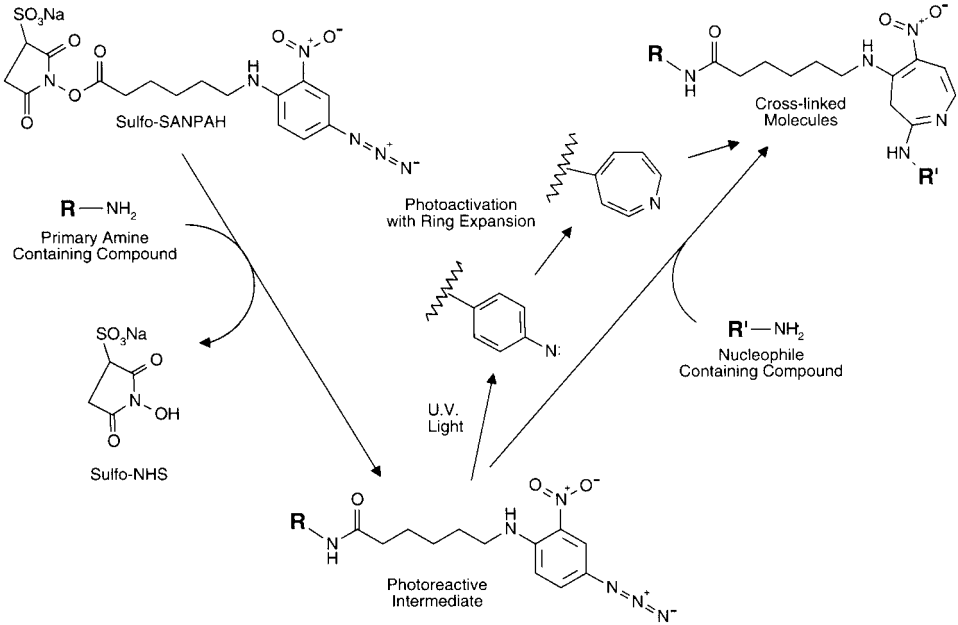


Figure 5.21 The reaction sequence of crosslinking with sulfo-SANPAH involves first derivatizing an amine-containing molecule using its NHS ester end to create an amide bond. Exposure to UV light then causes ring expansion to the dehydroazepine derivative, which can couple with amines to form the final conjugate.

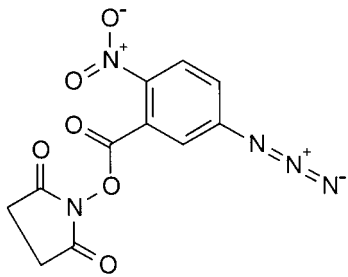
stable amide linkages. The photoreactive end is sensitive to long UV light, being selectively activated to a highly reactive nitrene or dehydroazepine intermediate. Either of these photolyzed species can couple to molecules within van der Walls contact, rapidly forming covalent bonds (Figure 5.21). The cross-bridge of SANPAH is a non-cleavable 6-aminohexanoic acid derivative, which provides a long spacer between conjugated molecules. The phenyl azide group also contains a nitro group on the ring that has the effect of increasing the wavelength of optimal photolysis. Exposure to light at a wavelength in the range of 320–350 nm promotes the photoreaction process. SANPAH is a water-insoluble crosslinker that will permeate membrane structures efficiently. The reagent should be dissolved in DMSO or DMF prior to addition of an aliquot to an aqueous reaction medium.

Reported applications of SANPAH include the crosslinking of ligand–receptor complexes *in situ* (Ballmer-Hofer *et al.*, 1982), preparing photoactivatable glycopeptide derivatives for site-specific labeling of lectins (Baenziger and Fiete, 1982), photoaffinity labeling of the *N*-formyl peptide receptor binding site of intact human polymorphonuclear leukocytes (Schmitt *et al.*, 1983), and the crosslinking of vasoactive intestin al peptide to receptors on intact human lymphoblasts (Wood and O'Dorisio, 1985).

A water-soluble version of this crosslinker also exists. Sulfo-SANPAH (sulfosuccinimidyl-6-(4′-azido-2′-nitrophenylamino)hexanoate) contains the negatively charged sulfonate group on its NHS ring, lending greater hydrophilicity to the compound. SANPAH and sulfo-SANPAH also have been used for investigations into endothelial cell spreading and adhesion (Wallace *et al.*, 2007), studying the behavior of pre-osteoblastic cells (Khatiwala *et al.*, 2006), and in the development of a real-time microscopic method for studying biomolecular interactions (Sasuga *et al.*, 2006).

3.5. ANB-NOS

N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) is a photoreactive, heterobifunctional crosslinker containing an amine-reactive NHS ester group (Thermo Fisher). Its cross-bridge is formed from a benzoic acid derivative, allowing molecules to be conjugated at relatively short 7.7 Å distances apart. The phenyl ring of ANB-NOS contains a nitro group that has the effect of shifting the optimal wavelength of activation to longer UV regions. The photoreaction is initiated by exposure to light in the range of 320–350 nm. Without the presence of the nitro group, activation would occur at much lower wavelengths, around 265–275 nm—wavelengths that potentially can damage biological molecules when exposed under high photon irradiation. ANB-NOS typically is used to label an amine-cont aining protein or molecule by its NHS ester end. The resultant derivative is allowed to interact with other molecules that potentially can bind specifically to it and photolyzed to effect the final conjugation, capturing any interacting partners (Figure 5.22).



ANB–NOS
N-5-Azido-2-nitrobenzoyloxy-
succinimide
MW 305.21
7.7 Å

Reported applications using this reagent include crosslinking of the aggregation state of cobra venom phospholipase A2 (Lewis *et al.*, 1977) and conjugation of the signal sequence of nascent preprolactin to a polypeptide of the signal recognition particle (Krieg *et al.*, 1986). In addition, ANB-NOS has been used to capture interacting proteins in a two-step process (Nadeau and Carlson, 2007), to study the self-association of the yeast TATA binding domain (Adams *et al.*, 2004), and to investigate the peptide binding cleft of major histocompatibility complex (MHC) class I molecules (Park *et al.*, 2003).

3.6. SAND

Sulfosuccinimidyl-2-(*m*-azido-*o*-nitrobenzamido)-ethyl-1,3′-dithiopropionate (SAND) is a heterobifunctional reagent containing an amine-reactive sulfo-NHS ester at one end and a photoreactive phenyl azide group on the other end (Thermo Fisher). The presence of the sulfonate group on the NHS ring lends water solubility to the reagent due to its negative charge in aqueous solutions. In addition, the phenyl azide group contains a nitro constituent which shifts the optimal range of photoactivation toward higher wavelengths—into the 320–350 nm region, thus decreasing the potential of photolytic damage to other sensitive groups that may be present during crosslinking. The extended cross-bridge of SAND (18.5 Å) provides a long spacer to accommodate even

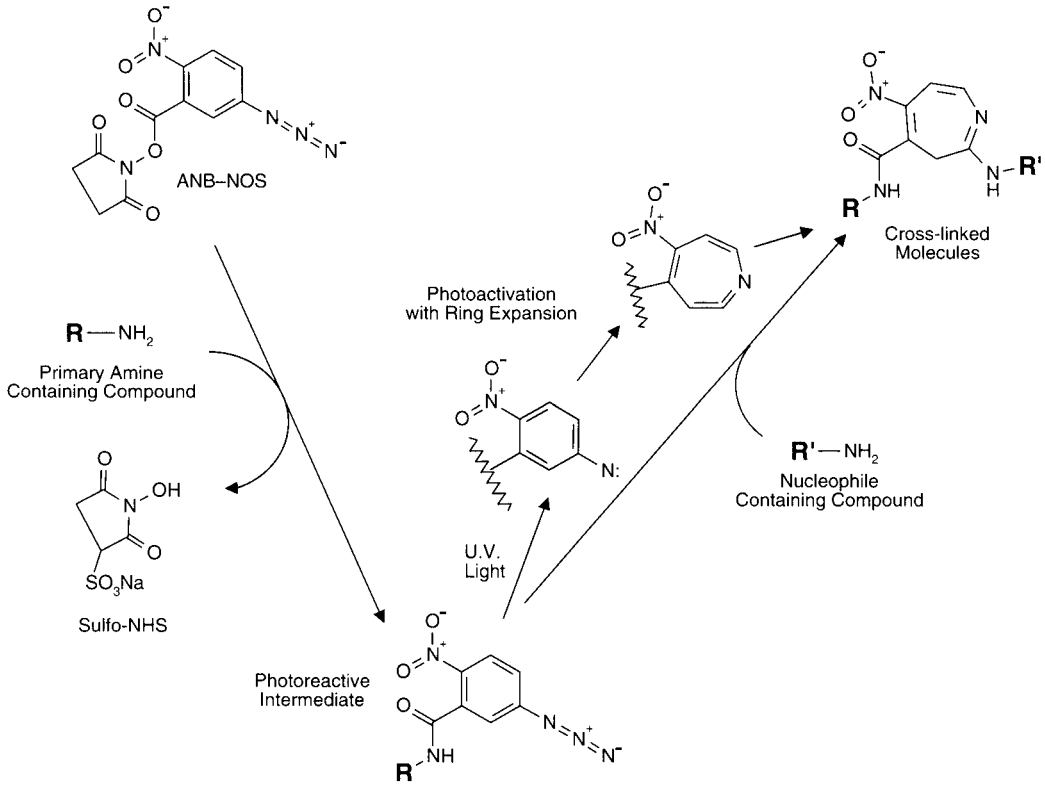
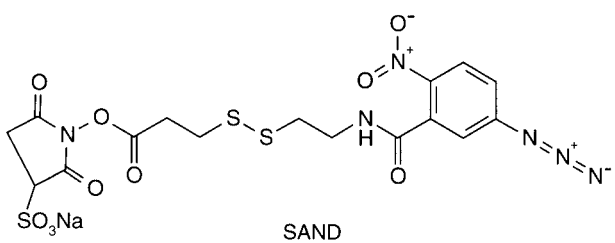


Figure 5.22 The NHS ester of ANB-NOS reacts with amines to form amide bonds. Subsequent photoactivation of the complex with UV light causes phenyl azide ring expansion and reaction with neighboring amines.

relatively distant sites between interacting molecules. The presence of a disulfide bond within the cross-bridge means that the reagent also is cleavable by the use of a disulfide reductant, allowing the potential for disruption of the crosslinks after purification of the conjugate.

In use, SAND is first reacted with an amine-containing protein or other molecule—being careful to protect the photoreactive group from inadvertent degradation by exposure to excessive room light or sun. The modified intermediate then is allowed to interact with a target molecule. Finally, the photolyzing process is done to effect a nonselective crosslink between the



SAND
Sulfosuccinimidyl-2-(*m*-azido-*o*-nitro-benzamido)-ethyl-1,3'-dithiopropionate
MW 570.52
18.5 Å

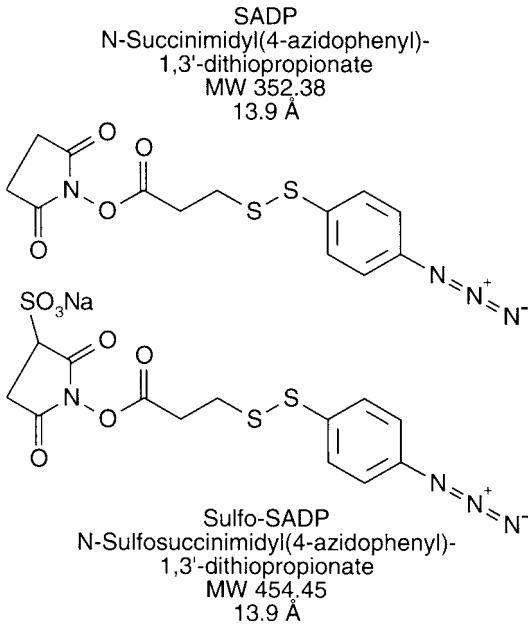
modified molecule and any target molecules within van der Waals distance to the crosslinker (Figure 5.23). Its use may be similar to that reported for sulfo-SANPAH, and its cleavability similar to that reported for SADP. For a more detailed discussion on the use of photoreactive crosslinkers to capture protein–protein interactions, see Chapter 28.

3.7. SADP and Sulfo-SADP

SADP, *N*-succinimidyl-(4-azidophenyl)1,3′-dithiopropionate, is a photoreactive heterobifunctional crosslinker that is cleavable by treatment with a disulfide reducing agent (Thermo Fisher). The crosslinker contains an amine-reactive NHS ester and a photoactivatable phenyl azide group, providing specific, directed coupling at one end and nonselective insertion capability at the other end.

SADP is first used to modify a protein via its amine groups through the reactive NHS ester end of the crosslinker. After allowing for interaction of the modified protein with target molecules, the photoreactive group is used to couple with any molecules within van der Waals distance. The photolysis reaction requires UV exposure in the range of 265–275 nm to effect the final linkage. The presence of the disulfide group in SADP's cross-bridge allows disruption of crosslinks with 50 mM DTT after the conjugation reaction is complete (Figure 5.24).

SADP is hydrophobic and should be dissolved in organic solvent prior to addition of a small aliquot to an aqueous reaction. Concentrated stock solutions can be prepared in dry DMSO or DMF. Final concentration of the organic solvent in a crosslinking reaction should not exceed about 10 percent to prevent protein precipitation or denaturation.



Reported applications of SADP include the crosslinking of con A to receptors oVn human erythrocyte membranes (Vanin and Ji, 1981), site-specific labeling of lectins using modified

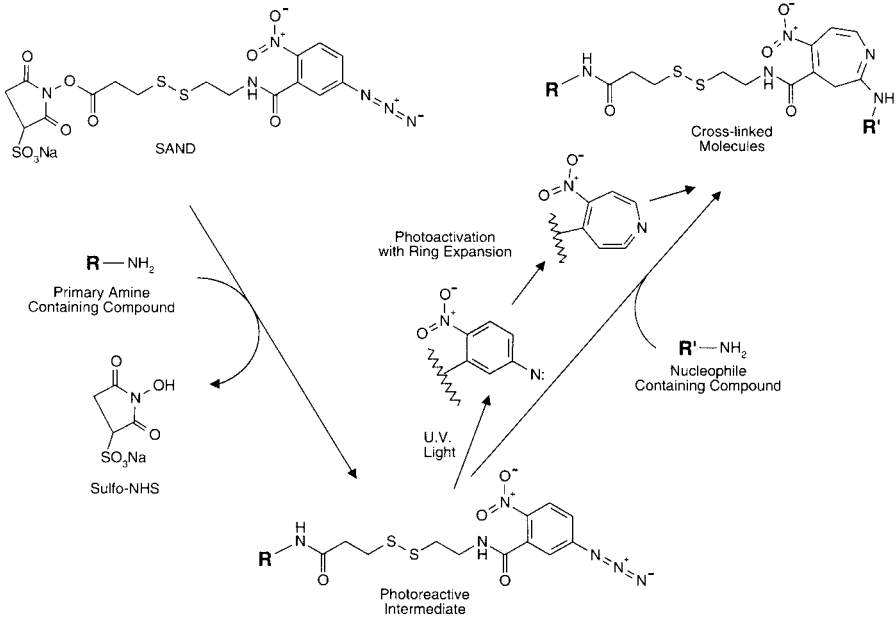


Figure 5.23 SAND can be used to modify amine-containing molecules, and then photo-initiate crosslinking to another amine-containing molecule via a ring-expansion process. The conjugates may be disrupted by reduction of the cross-bridge disulfide with DTT.

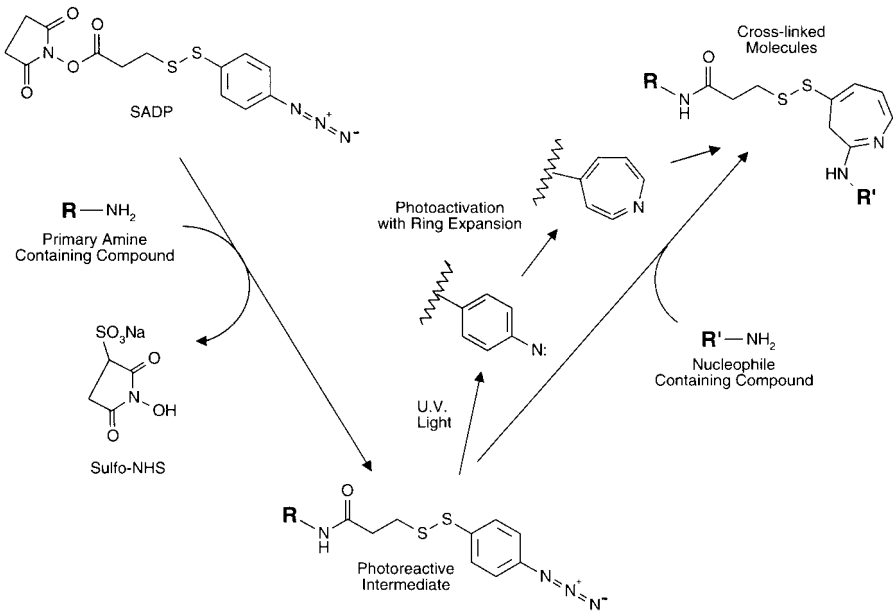


Figure 5.24 SADP reacts with amines via its NHS ester end to produce amide bonds. The modified molecule then may be photoactivated to create a nucleophile-reactive dehydroazepine intermediate able to covalently couple with amine-containing compounds.

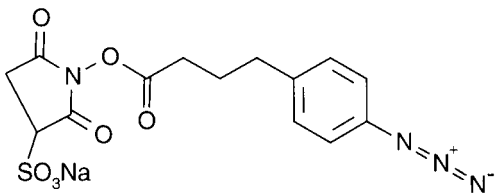
glycopeptides (Baenziger and Fiete, 1982), conjugation of a mouse cell–surface polypeptide with a Sendai virion envelope on newly infected cells (Zarling *et al.*, 1982), and crosslinking of platelet glycoprotein Ib (Jung and Moroi, 1983).

Sulfo-SADP is a water-soluble analog of SADP which contains a negatively charged sulfonate group on its NHS ring. The reagent may be added directly to aqueous reaction mixtures without prior dissolution in an organic solvent. Concentrated stock solutions prepared in water should be used immediately to prevent extensive hydrolysis of the sulfo-NHS ester group.

SADP or sulfo-SADP also have been used to study the phenylalanine-methionine-arginine-phenylalanine-amide-activated sodium channel (Coscoy *et al.*, 1998), various apolipoprotein E isoforms (Mann *et al.*, 1995), the high-affinity phenylalkylamine Ca^{2+} antagonist binding protein from guinea pig (Moebius *et al.*, 1994), the interaction of non-histone proteins with nucleosome core particles (Reeves and Nissen, 1993), and the interactions among cytochromes P-450 in the endoplasmic reticulum (Alston *et al.*, 1991). See Chapter 28 for methods of using photoreactive heterobifunctional crosslinkers to study protein interactions.

3.8. Sulfo-SAPB

Sulfo-SAPB, sulfosuccinimidyl-4-(*p*-azidophenyl)butyrate, is a photoreactive heterobifunctional reagent containing an amine-reactive sulfo-NHS ester at one end (Thermo Fisher). The crosslinker is similar in design to sulfo-HSAB (Section 3.3, this chapter), but containing a 3-carbon-longer cross-bridge. The sulfo-NHS ester provides water solubility to the reagent due to the negative charge of the sulfonate group. The phenyl azide end can be photolyzed by exposure to UV light in the wavelength range of 265–275 nm (Figure 5.25). Although there are no reported applications for the crosslinker, its reactivity and use is similar to that of sulfo-HSAB. The commercial availability of the reagent provides additional options for spacer length to study the interactions between two proteins or other molecules.



Sulfo-SAPB
Sulfosuccinimidyl-
4-(*p*-azidophenyl)butyrate
MW 404.32
12.8 Å

3.9. SAED

Sulfosuccinimidyl-2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3′-dithiopropionate (SAED) is a photoreactive heterobifunctional crosslinking agent that also contains a fluorescent group (Thermo Fisher). The sulfo-NHS ester end of the reagent reacts with primary amines in proteins and other molecules to form stable amide linkages. The photoreactive end is an AMCA

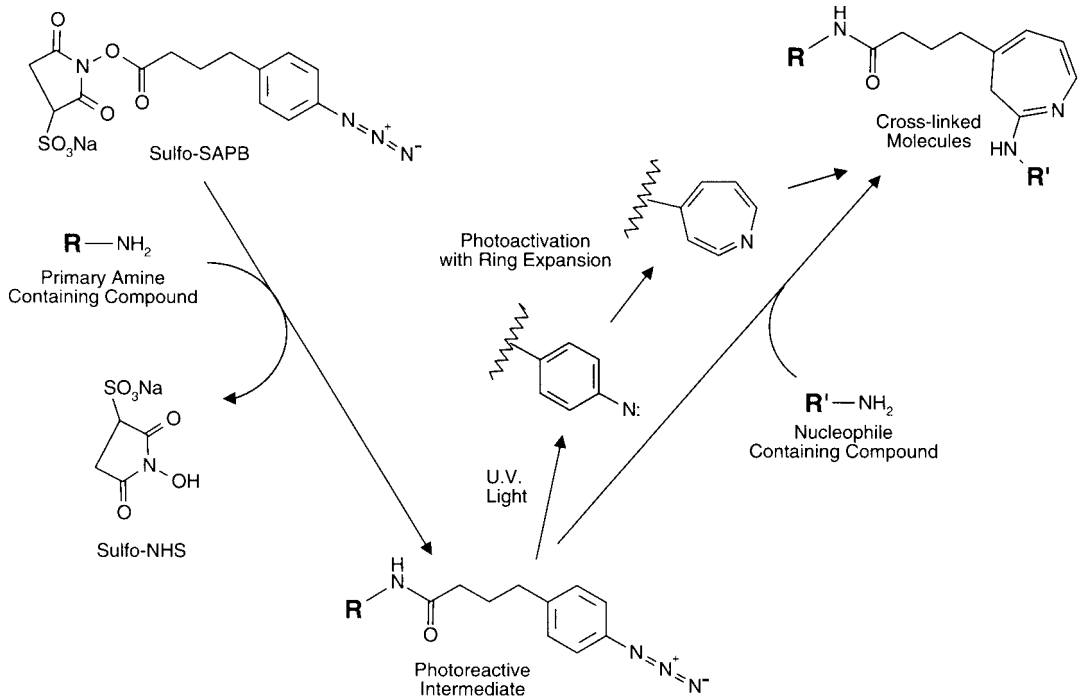
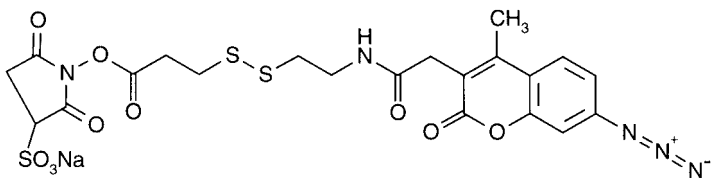


Figure 5.25 The reaction of sulfo-SAPB with an amine group is done first to form an amide bond derivative through its NHS ester end. Subsequent exposure to UV light causes the phenyl azide group to ring-expand to a highly reactive dehydroazepine, which can couple to nucleophiles, such as amines.

derivative (Chapter 9, Section 3) containing a light-sensitive azide group on the aromatic ring. Photolyzing with light in the long UV range will result in nonselective bond formation with nucleophiles and active carbon–hydrogen bonds within van der Waals distance (Figure 5.26).



SAED
Sulfosuccinimidyl-2-(7-azido-4-methyl
coumarin-3-acetamide)ethyl-1,3'-dithiopropionate
MW 621.6
23.6 Å

SAED is a relatively large crosslinker containing a long (22.5 Å) cross-bridge. The central portion of its cross-bridge contains a disulfide bond, making the reagent susceptible to cleavage with disulfide reducing agents. The aromatic character of the coumarin derivative creates a maximal UV absorptivity at 327 nm with an extinction coefficient of 18,200 M^{-1} cm^{-1} for a 1 mg/ml solution in acetonitrile:water (15:2 v/v). The extinction coefficient at 298 nm for the same concentration of SAED in the identical solvent is 13,625 M^{-1} cm^{-1}.

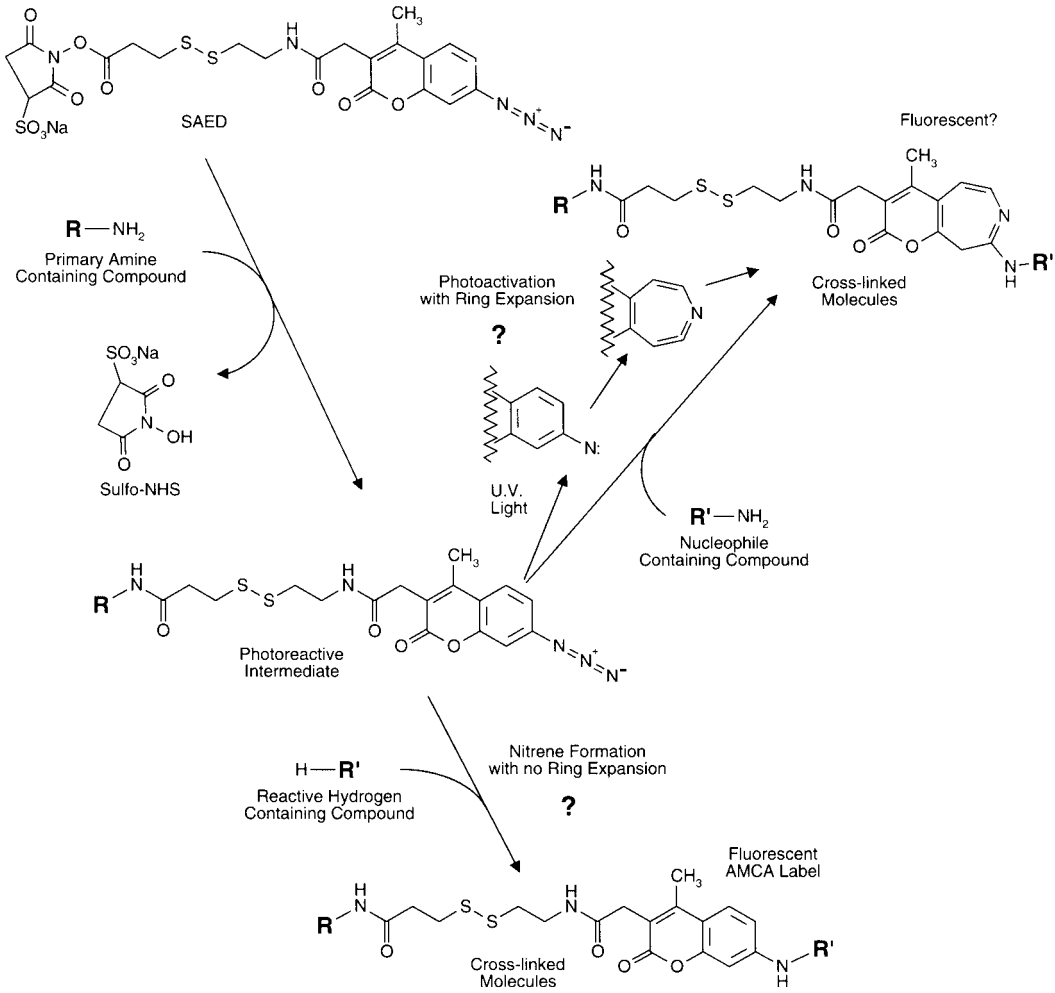


Figure 5.26 SAED can be used to modify amine-containing molecules through its NHS ester end. Subsequent exposure to UV light causes bond formation with nearby nucleophilic groups, such as amines. The photosensitive phenyl azide group is created on the aromatic ring of an AMCA fluorophore. Before photoactivation, the azide group makes the crosslinker nonfluorescent. After photoactivation, however, the azide group is either lost by N_2 generation or couples to a target molecule. Either way, the AMCA portion becomes fluorescent to allow tracking of the conjugate. The photoreaction may occur through ring expansion to an intermediate dehydroazepine or might happen through nitrene formation.

SAED contains a sulfo-NHS ester with a negatively charged sulfonate group on its ring. The presence of this negative charge does lend some expected water solubility to the reagent (3 mg/ml at room temperature), but because of the reagent's large size it does not provide the same water solubility benefits as with other smaller crosslinkers. It is also sparingly soluble in acetonitrile (2.5 mg/ml), but only if a small amount of water is present (15:2 acetonitrile:water, v/v). However, SAED is very soluble in DMSO and DMF (about 50 mg/ml). Stock solutions may be prepared in dry DMSO or DMF while maintaining fairly good stability of the

reagent's functional groups. The addition of a small quantity of these stock solutions to an aqueous reaction medium facilitates the amine modification process via the sulfo-NHS ester end of the crosslinker. The final concentration of organic solvent in the aqueous reaction should not exceed 10 percent to avoid protein denaturation and precipitation. Protect all solutions of the crosslinker from light to prevent premature activation of the photoreactive group.

The coumarin derivative of SAED is not fluorescent until the photolysis reaction is initiated. A protein modified with SAED will fluoresce after activation with UV light whether or not the photoreactive end actually couples to the intended target, since breakdown of the azide group on the ring is all that is required to initiate fluorescence. Thus, the level of SAED incorporation into a macromolecule may be assessed by the resultant coumarin fluorescence after separation of the derivative from excess reagent. Native AMCA has an excitation optimum at 345–350 nm and an emission wavelength range of 440–460 nm. The quantum yield of SAED may change somewhat upon its attachment to macromolecules due to fluorescent quenching; however, the coumarin tag will still remain fluorescently active even after crosslinking.

Since the crosslinker is cleavable, SAED provides a means of fluorescent transfer of the coumarin tag to a second molecule which interacts with the initially modified protein (Figure 5.27). For example, soybean trypsin inhibitor (STI) was labeled with SAED and then allowed to interact with trypsin. After photoreactive crosslinking of the two interacting molecules, the complex was reduced with DTT, breaking the conjugate and transferring the fluorescent tag to trypsin near the STI binding site (Thevenin *et al*., 1991). This type of fluorescent label transfer reagent is important for studying unknown interacting proteins, because the unknown protein can be detected and isolated by the tag after cleavage of the complex.

In another study, SAED was used to investigate the role of the foot protein moiety of the triad and its relationship to Ca^{2+} release from sarcoplasmic reticulum (Kang *et al*., 1991). Modification of poly-L-lysine (a Ca^{2+} release inducer) and neomycin with the crosslinker was done followed by subsequent incubation with the foot protein and photoreactive conjugation. Cleavage of the crosslinks with a disulfide reductant allowed transfer of the fluorescent tag to the foot protein in areas near the binding sites. Fluorescent monitoring of conformational changes within the protein upon varying the Ca^{2+} concentration was then possible.

Since the photoreactive crosslinking step with SAED occurs rapidly upon exposure to even bright light within the visible spectrum, UV lamps are not required. However, special care should be taken to protect the reagent from exposure to light before the photolysis reaction is initiated. The solid should be stored in amber bottles and any stock solutions prepared in organic solvent should be wrapped to exclude light. In addition, the initial derivatization of an amine-containing molecule should be done in the dark in wrapped containers.

Additional applications of SAED include study of the ryanodine receptor (Yano *et al*., 2005; Mochizuki *et al*., 2007) and investigating the protein organization of the postsynaptic density (Liu *et al*., 2006).

3.10. Sulfo-SAMCA

Sulfo-SAMCA, sulfosuccinimidyl-7-azido-4-methylcoumarin-3-acetate, is a heterobifunctional reagent similar in design to SAED (Section 3.9, this chapter) (Thermo Fisher). One end of the crosslinker contains an amine-reactive sulfo-NHS ester, while the other end is an AMCA derivative (Chapter 9, Section 3) that contains a photosensitive phenyl azide group. Unlike

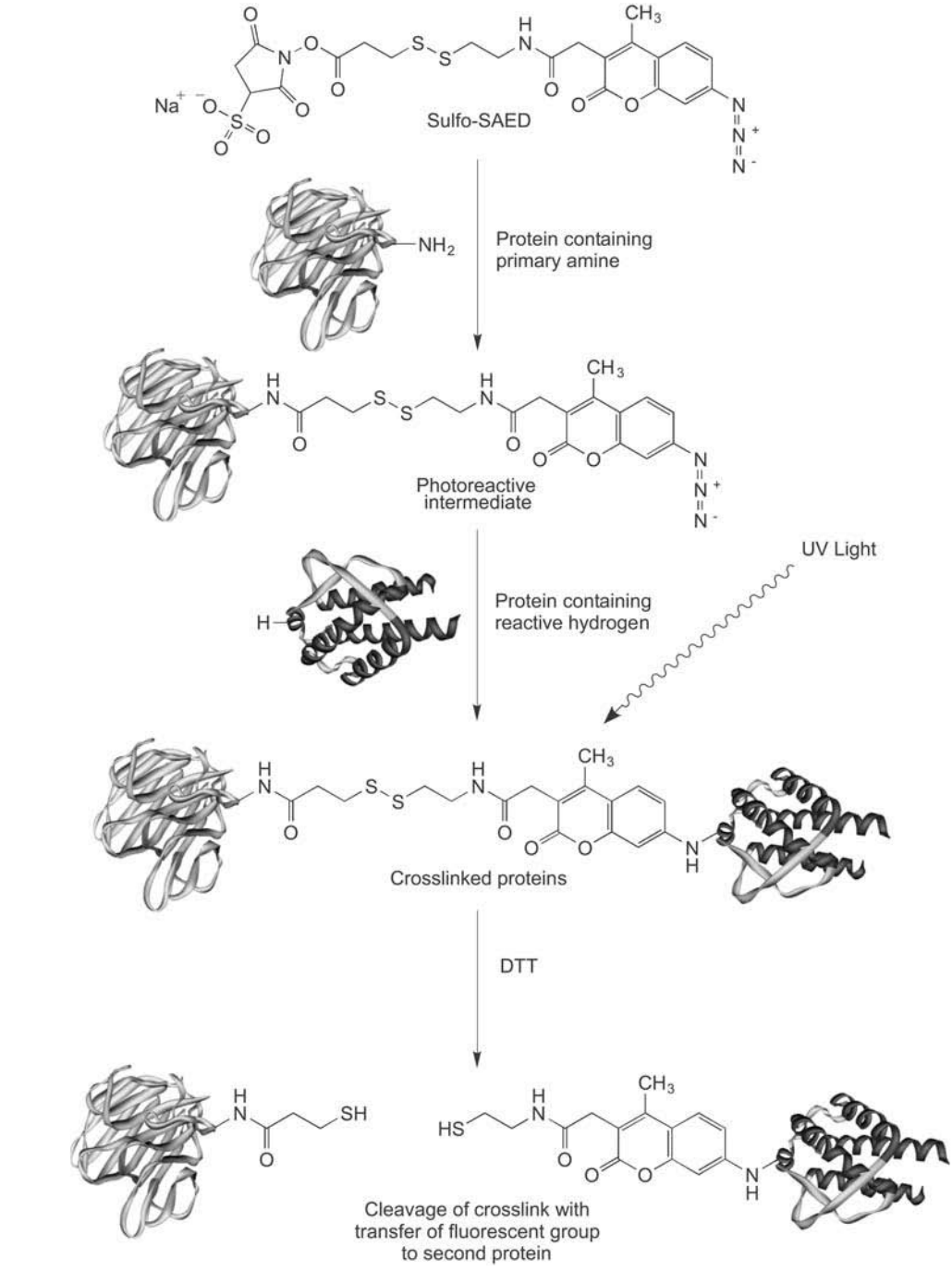


Figure 5.27 SAED may be used to transfer the fluorescent AMCA label from the first molecule modified with the crosslinker to the second molecule crosslinked with it by reduction of its internal disulfide bond. Thus, unknown target molecules may be fluorescently tagged to follow them *in vivo*.

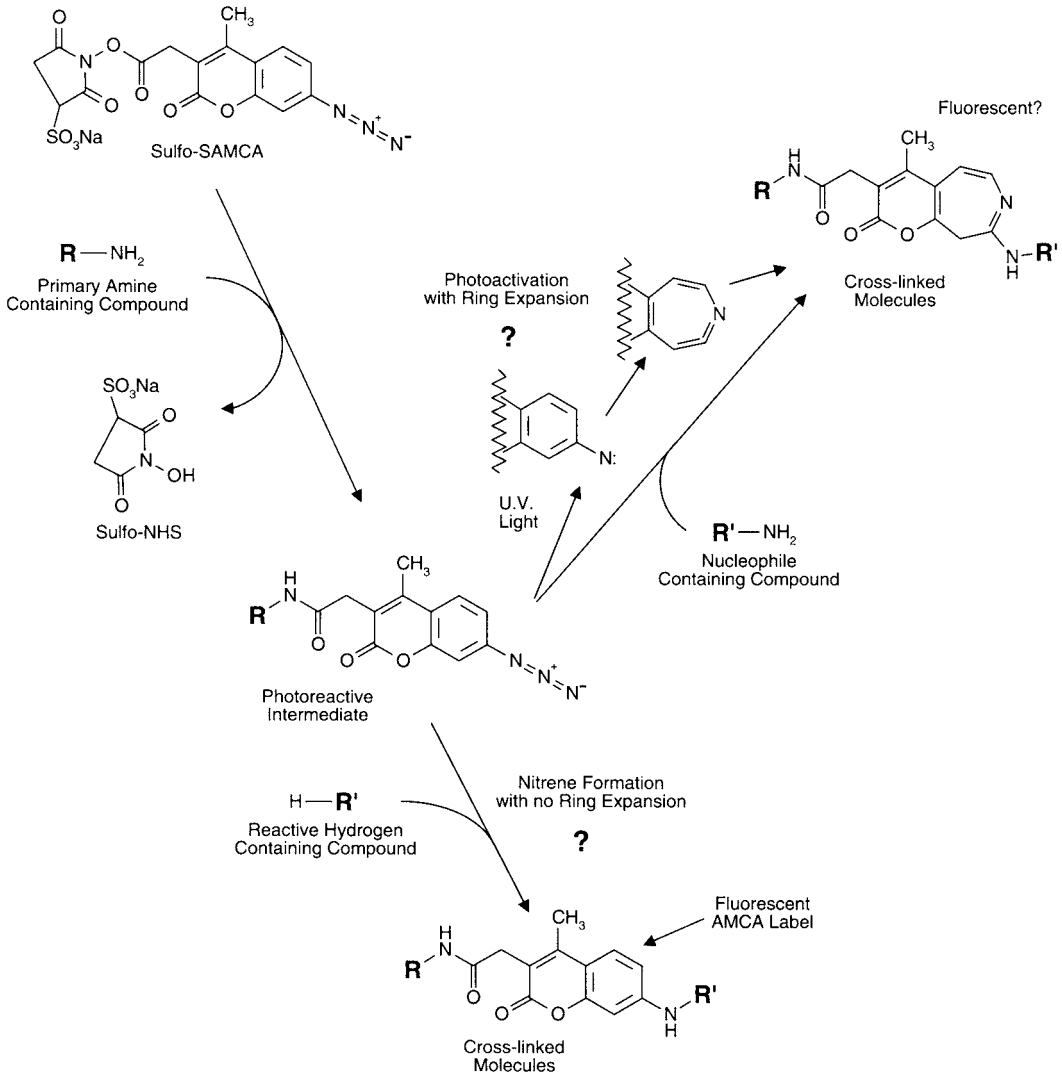
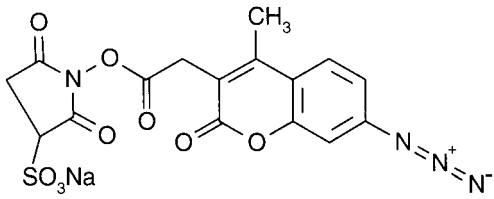


Figure 5.28 Sulfo-SAMCA can be used to modify amine-containing molecules through its NHS ester end. Subsequent exposure to UV light causes bond formation with nearby nucleophilic groups, such as amines. The photosensitive phenyl azide group is created on the aromatic ring of an AMCA fluorophore. Before photoactivation, the azide group makes the crosslinker nonfluorescent. After photoactivation, however, the azide group is either lost by N_2 generation or couples to a target molecule. Either way, the AMCA portion becomes fluorescent to allow tracking of the conjugate. The photoreaction may occur through ring expansion to an intermediate dehydroazepine or might happen through nitrene formation.

SAED, however, sulfo-SAMCA contains a short non-cleavable cross-bridge (12.8 Å) where the active ester functionality is constructed directly off the carboxylate group of AMCA without any other intervening spacer groups. Conjugated molecules will retain the fluorescent label, thus providing detectability to the complexes formed (Figure 5.28). However, since crosslinks

formed with this reagent are not cleavable, sulfo-SAMCA cannot function as a fluorescent label transfer agent in the fashion of SAED.

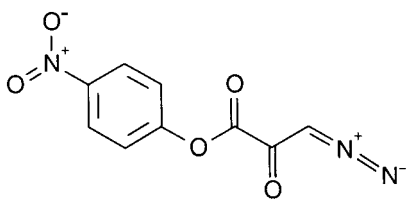


Sulfo-SAMCA
Sulfosuccinimidyl-7-azido-
4-methylcoumarin-3-acetate
MW 458.34
12.8 Å

3.11. *p*-Nitrophenyl Diazopyruvate

Diazopyruvates represent a unique class of photoreactive reagents that are not often used in heterobifunctional crosslinker design. The *p*-nitrophenyl ester derivative of diazopyruvate provides amine-reactive, acylating potential, while the photosensitive group can be activated with UV light to generate reactive aldehydes. More specifically, the diazo functionality can be photolyzed by exposure to irradiation at 300 nm, forming a highly reactive carbene which can undergo a Wolff rearrangement that produces a ketene amide intermediate. In the presence of a nucleophilic species on a target molecule, the ketene can undergo an acylation reaction to form a stable malonic acid derivative. The photolyzed product thus can couple to hydrazide- or amine-containing targets to form covalent linkages (Figure 5.29).

p-nitrophenyl diazopyruvate (Invitrogen) is relatively insoluble in water or aqueous buffers, but may be pre-dissolved in DMF before adding an aliquot of the stock solution to an aqueous reaction mixture. All solutions of the reagent should be carefully protected from light to prevent premature photolysis. *p*-nitrophenyl diazopyruvate has an absorbance maximum at 390 nm with a molar extinction coefficient of about 19,000 $M^{-1}\,cm^{-1}$ in methanol.



pNPDP
p-Nitrophenyl diazopyruvate
MW 235

p-nitrophenyl diazopyruvate has been used in the photoreactive crosslinking of calmodulin with adenylate cyclase from bovine brain (Harrison *et al.*, 1989), to crosslink aldolase (Goodfellow *et al.*, 1989), as a bonding agent for tissue containing type-I collagen (Givens

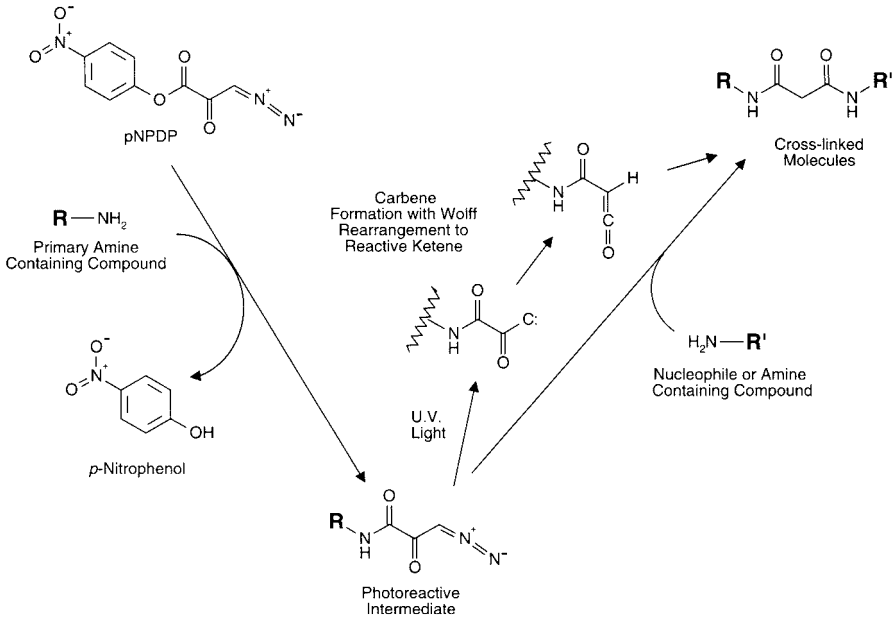
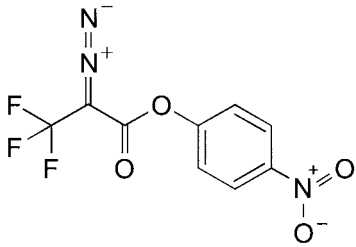


Figure 5.29 pNPDP reacts with amine-containing compounds by its *p*-nitrophenyl ester group to form amide bonds. After photoactivation of the diazo derivative with UV light, a Wolff rearrangement occurs to a highly reactive ketene intermediate. This group can couple to nucleophiles such as amines.

et al., 2003) or to bond to corneal tissue (Timberlake *et al.*, 2005), and in the photoreactive coupling of DNA to paramagnetic beads (Penchovsky *et al.*, 2000).

3.12. PNP-DTP

PNP-DTP, *p*-nitrophenyl-2-diazo-3,3,3-trifluoropropionate, is a photoreactive heterobifunctional crosslinker that contains an amine-reactive group on one end and a photosensitive diazo group on the other (Chowdhry *et al.*, 1976) (Thermo Fisher). *p*-nitrophenyl esters react similarly



PNP-DTP;
p -Nitrophenyl-2-diazo-
3,3,3-trifluoropropionate
Mol. Wt.: 275.14

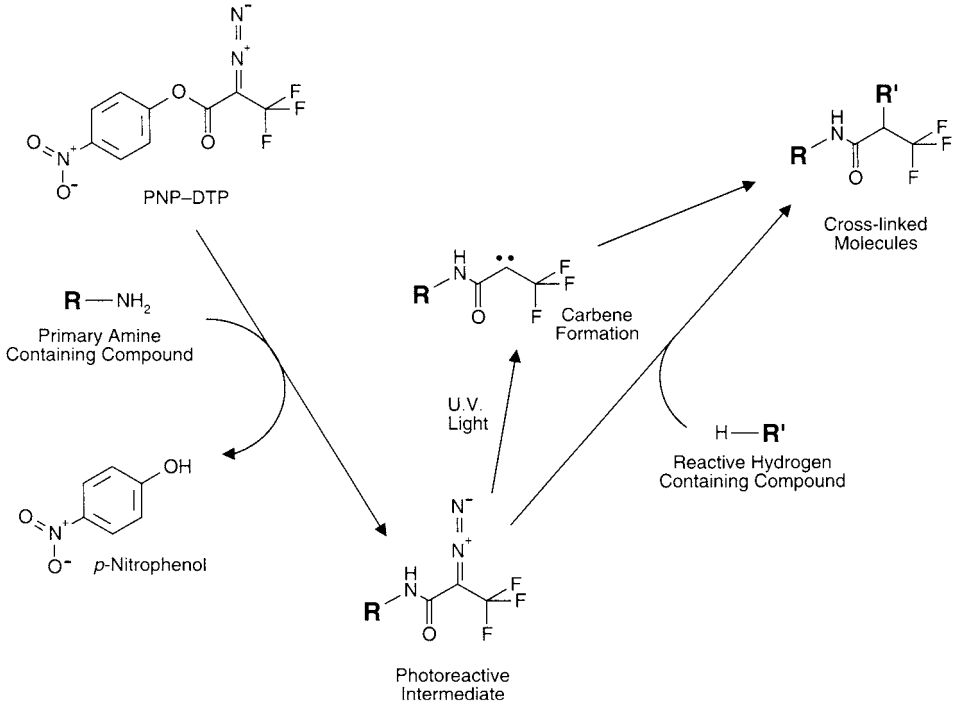


Figure 5.30 PNP-DTP can modify amine-containing molecules through its *p*-nitrophenyl ester group to form amide bonds. Exposure of its photosensitive diazo group with UV light generates a highly reactive carbene that can insert into active C—H or N—H bonds.

to NHS esters (Chapter 4, Section 1 and Chapter 2, Section 1.4) but in this case, with *p*-nitrophenol as the leaving group upon reaction with a nucleophile. Amine-containing target molecules such as proteins can be modified with this reagent to form amide bond derivatives possessing photoactivatable functionalities. The reagent is small enough to probe deep within the active centers of receptor molecules and other sites of biomolecular interactions (Figure 5.30).

PNP-DTP has been used to photoaffinity label the thyroid hormone nuclear receptors in intact cells by preparing a derivative of 3,5,3′-triiodo-L-thyronine with the crosslinker (Pascual *et al.*, 1982; Casanova *et al.*, 1984). Effective photoreactive conjugation was found to occur after irradiation with UV light at 254 or 310 nm.

4. Sulfhydryl-Reactive and Photoreactive Crosslinkers

The benefits of nonselective photoreactive crosslinking can be merged with the directed coupling ability of sulfhydryl-reactive functionalities to create heterobifunctional reagents possessing greater utility than the standard amine and photoreactive agents discussed previously. Having a sulfhydryl-reactive group on one end of the crosslinker allows the initial conjugation to take place at more discrete sites on proteins and other molecules before irradiation to effect the final photosensitive reaction.

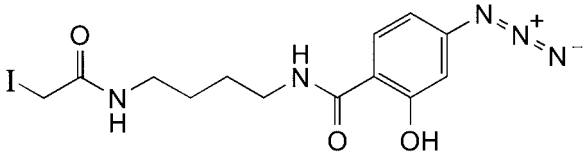
The following reagents contain a variety of sulfhydryl-reactive groups, including iodoacetyl derivatives, maleimide compounds, and pyridyl disulfide chemistries. The iodoacetyl and maleimide functions form permanent thioether bonds with target molecules containing free sulfhydryls. The pyridyl disulfide derivative reacts with —SH groups to form reversible disulfide linkages, which can be cleaved with disulfide reducing agents like DTT.

The photoreactive end of the following crosslinkers also varies from the traditional aryl azide group to the newer benzophenone and fluorinated aryl azide derivatives. The fluorinated phenyl azide functional groups photolyze to true nitrenes without the ring-expansion side reaction characteristic of aryl azides. The result is that fluorinated aryl azides more effectively insert into active carbon–hydrogen bonds, rather than potentially undergoing nucleophilic reactions like phenyl azides. In addition, benzophenone groups generally have higher degrees of bond formation with the intended target molecule compared to the yields obtained using traditional phenyl azides, due to their ability to be repeatedly photolyzed without breakdown of the precursor to an inactive form.

The number of commercially available crosslinkers for sulfhydryl and photoreactive conjugations provides enough variety to design successful experiments in photolabeling, such as studying active centers and macromolecular interactions.

4.1. ASIB

ASIB, 1-(*p*-azidosalicylamido)-4-(iodoacetamido)butane, is a heterobifunctional crosslinker containing a sulfhydryl-reactive iodoacetyl group on one end and a photosensitive phenyl azide group on the other end (Thermo Fisher). The phenyl azide ring is substituted with a ring-activating hydroxyl group which provides the ability to radioiodinate the compound before the conjugation reaction is performed. Since both the iodoacetyl and phenyl azide functionalities are relatively stable in aqueous solutions, the steps involved in iodination and crosslinking do not detrimentally affect the subsequent reactivity of the reagent. All operations should be done protected from light, however, to prevent premature photolysis before the desired crosslinking reaction is initiated. The cross-bridge of the reagent provides an 18.8 Å spacer between crosslinked molecules.



ASIB;
1-(p-Azidosalicylamido)-
4-(iodoacetamido)butane
Mol. Wt.: 417.20
18.8 Å

The reaction of ASIB with sulfhydryl-containing molecules can be done at mildly alkaline pH with excellent specificity. Higher pH conditions may cause cross-reactivity with amines.

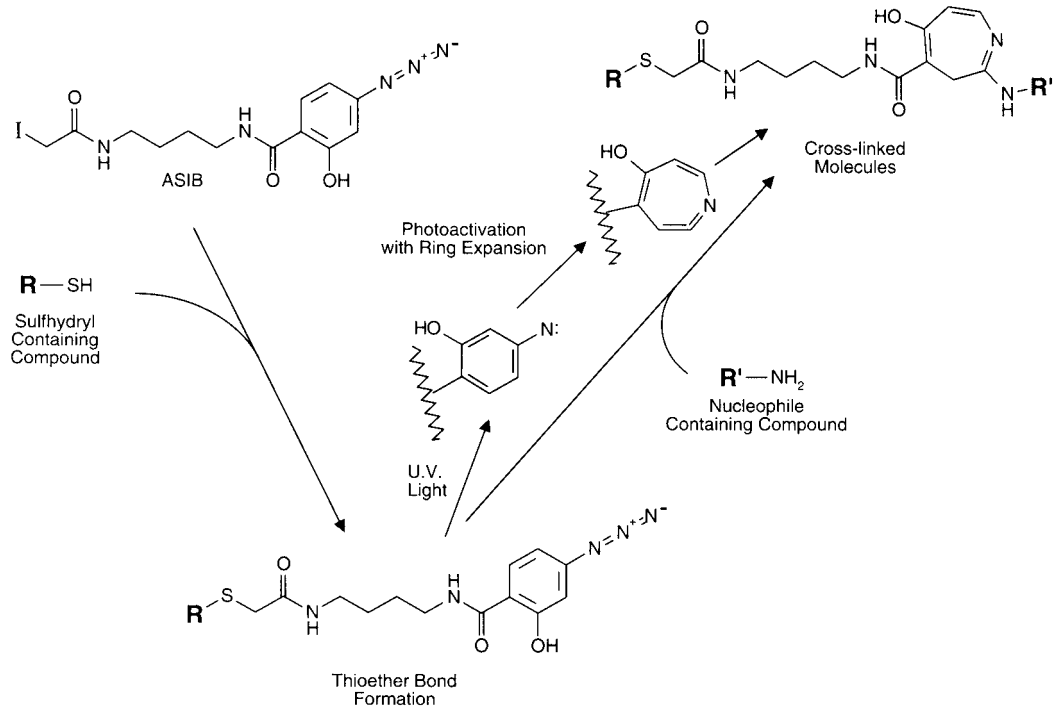


Figure 5.31 ASIB can react with sulfhydryl-containing molecules through its iodoacetate group to form thioether linkages. Subsequent exposure to UV light causes a ring-expansion process to occur, creating a highly reactive dehydroazepine intermediate that can couple to amine-containing molecules.

Photolyzing with UV light may result in immediate reaction of the nitrene intermediate with a target molecule within Van der Waals distance, or may result in ring expansion to the nucleophile-reactive dehydroazepine. The ring-expanded product is reactive primarily with amine groups (Figure 5.31).

4.2. APDP

APDP, *N*-[4-(*p*-azidosalicylamido)butyl]-3′-(2′-pyridyldithio) propionamide, is a radioiodinatable, heterobifunctional crosslinking agent that contains a sulfhydryl-reactive pyridyl disulfide group on one end and a photosensitive phenyl azide on the other end (Thermo Fisher). Radioiodinatable crosslinkers eliminate the need to radiolabel one of the reacting proteins, thus avoiding potential activity losses due to modification of important residues (Chapter 12, Section 5). They also allow radiolabeling of unknown target molecules which interact with the initially modified protein. APDP reacts with sulfhydryl-containing proteins and other molecules to form a reversible disulfide bond. If the crosslinker is radiolabeled prior to conjugation, cleavage of the disulfide group with DTT after crosslinking effectively transfers the iodinated portion to the secondary, photocoupled protein. This radiolabel transfer process allows tracking of a specific receptor or other interacting species after conjugation with its complementary

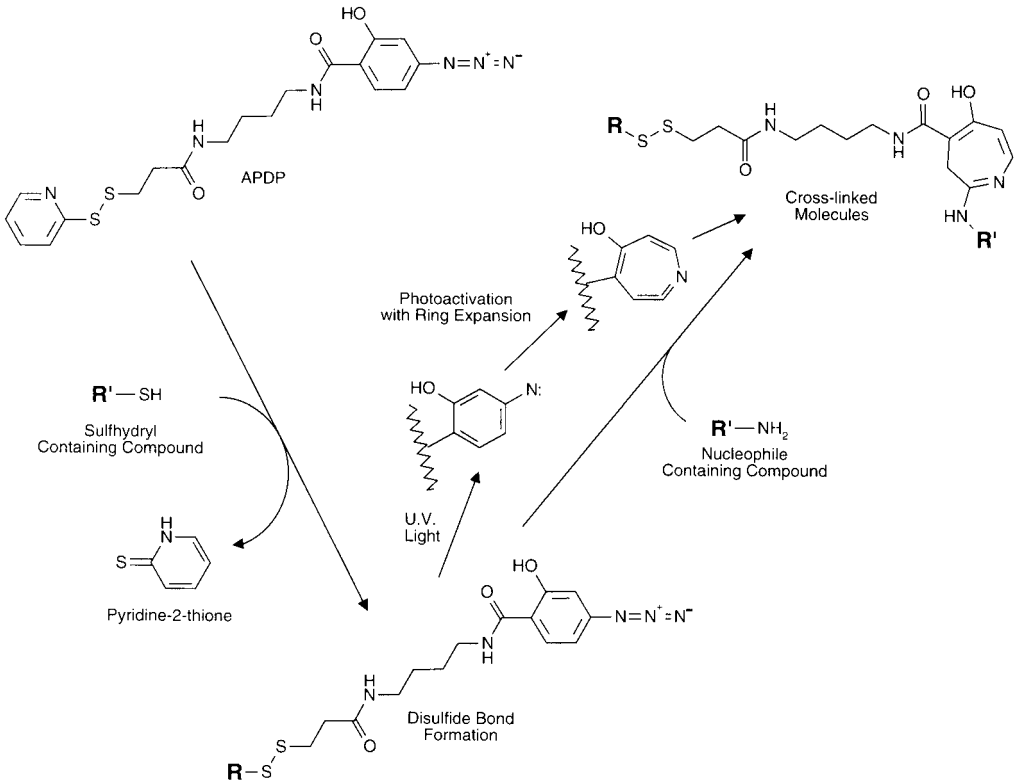
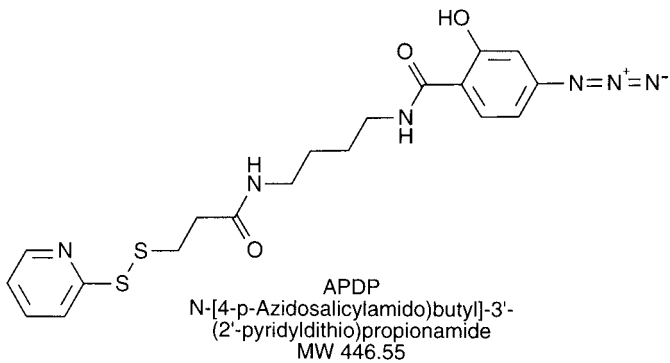


Figure 5.32 APDP can modify sulfhydryl-containing compounds through its pyridyl disulfide group to form disulfide bonds. Its phenyl azide end then can be photolyzed with UV light to couple with nucleophiles via a ring-expansion process. The disulfide group of the crosslink can be selectively cleaved using DTT.

ligand (Figure 5.32). APDP thus falls into the general category of label transfer reagents that can be used to study protein interactions (Chapter 28).



The reactions of APDP are similar to that of the reported compound *N*-(4-azidophenyl) thiophthalimide, a non-radioiodinatable crosslinker (Moreland *et al.*, 1982). Both the phenyl

azide group and the pyridyl disulfide portion are stable in aqueous environments prior to the crosslinking reaction. The initial modification with a sulfhydryl-containing protein should be done protected from light to preserve the activity of the photosensitive group. Avoid, also, in the reaction medium disulfide reducing agents that can react with the pyridyl disulfide group as well as inactivate the phenyl azide portion.

The cross-bridge of APDP provides a long, 21.02 Å spacer that is able to reach distant points between two interacting molecules. Cleavage of the crosslink with a disulfide reducing agent regenerates the original sulfhydryl-modified protein without leaving any other chemical groups behind. The remainder of the crosslinker stays attached to the second, interacting protein.

APDP is soluble in DMSO and DMF, but almost insoluble in acetone or water. Stock solutions may be prepared in DMSO or DMF and a small aliquot added to an aqueous reaction mixture. Do not exceed 10 percent organic solvent in the buffered reaction. Both functionalities of APDP will react in a variety of salt conditions and pH values. For reaction with a sulfhydryl-containing protein, a buffer at physiological pH containing a chelating agent to protect the free sulfhydryl groups from metal-catalyzed oxidation is recommended (i.e., 0.01–0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA).

Iodination of the crosslinker may be done according to the procedures discussed in Chapter 12, Section 5, or performed similar to that described for SASD (Section 3.2, this chapter).

4.3. Benzophenone-4-iodoacetamide

A photoreactive group consisting of a benzophenone residue photolyzes upon exposure to UV light to give a highly reactive triplet-state ketone intermediate (Walling and Gibian, 1965). Similar to the reactive nitrene of photolyzed phenyl azides, the energized electron of an activated benzophenone can insert in active hydrogen–carbon bonds and other reactive groups to give covalent linkages with target molecules. Unlike phenyl azides, however, the decomposition or decay of the photoactivated species does not yield an inactive compound. Instead, benzophenones that have become deactivated without forming a covalent bond can be once again photolyzed to an active state. The results of this multiple-activation characteristic are more than one chance to form a crosslink with the intended target and much higher yields of photo-crosslinking.

The heterobifunctional crosslinker benzophenone-4-iodoacetamide is a photoreactive reagent containing a sulfhydryl-reactive iodoacetyl derivative at one end and a benzophenone group on the other end (Hall and Yalpani, 1980; Tao *et al.*, 1984; Lu and Wong, 1989) (Invitrogen). The iodoacetyl group has similar reactivity to the same group on the heterobifunctional reagent SIAB (Section 1.5, this chapter). Under alkaline conditions (pH 8–9), the iodoacetyl reaction is highly specific for sulfhydryl residues in proteins and other molecules, forming stable thioether linkages. The initial modification reaction of sulfhydryl-containing compounds should be done protected from light to avoid premature photolysis of the benzophenone functionality. Also, avoid thiol-containing reducing agents in the sample, as these will react with the iodoacetyl group. After purification of the benzophenone-modified protein from excess reagent (by dialysis or gel filtration), it is mixed with a sample containing a second target molecule (e.g., a cell lysate) to allow an interaction to take place, and then photolyzed with UV light to effect the

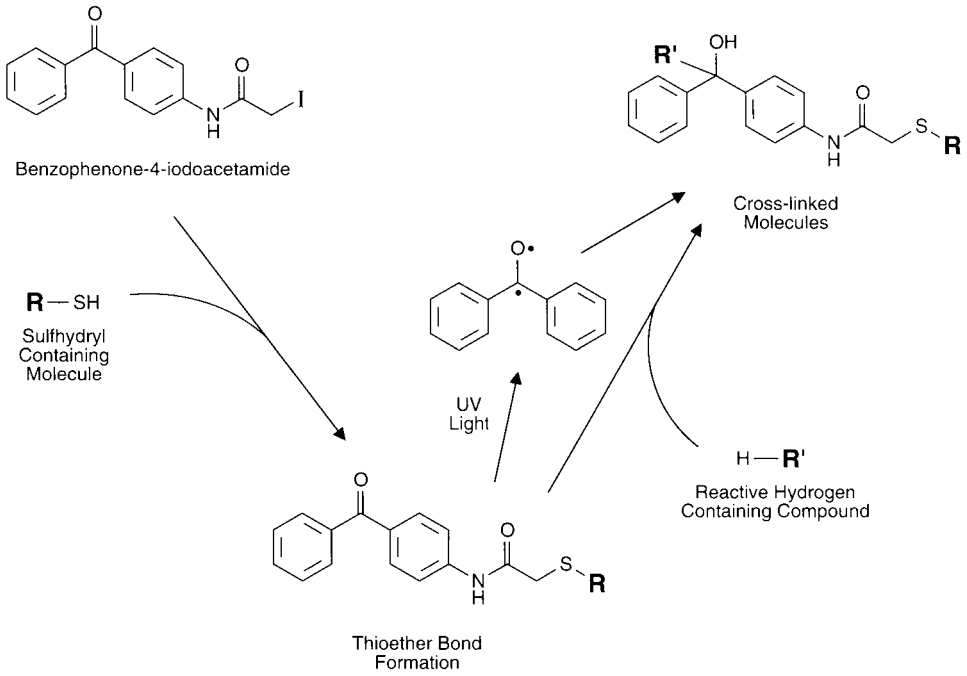
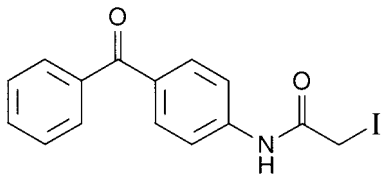


Figure 5.33 Benzophenone-4-iodoacetamide reacts with sulfhydryl-containing compounds to give thioether linkages. Subsequent photoactivation of the benzophenone residue gives a highly reactive triplet-state ketone intermediate. The energized electron can insert in active C—H or N—H bonds to give covalent crosslinks.

final crosslink (Figure 5.33). Since repeated photolysis of the benzophenone species is possible, the yield of such conjugation reactions can be significantly higher than using other photoreactive groups. One report indicated that crosslinking with chymotrypsin approached 100 percent efficiency (Campbell and Gioannini, 1979).



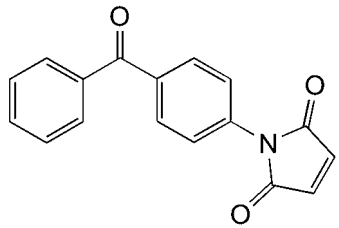
Benzophenone-4-iodoacetamide
MW 365

Benzophenone-4-iodoacetamide is water-insoluble and should be pre-dissolved in DMF or another organic solvent prior to adding an aliquot to an aqueous reaction mixture. Stock solutions may be prepared and stored successfully if protected from light.

Benzophenone-4-iodoacetamide has been used to study the 100-KDa U5 snRNP protein (hPrp28p) and its interactions (Ismaili *et al.*, 2001).

4.4. Benzophenone-4-maleimide

Benzophenone-4-maleimide is a heterobifunctional photoreactive crosslinker that has sulfhydryl reactivity similar to benzophenone-4-iodoacetamide discussed in the previous section (Invitrogen). In this case, the sulfhydryl-reactive portion is provided by the presence of a maleimide group that couples to thiols by addition to the double bond (Chapter 2, Section 2.2). The maleimide group is specific for sulfhydryls under physiological conditions, and the reaction results in a thioether linkage that is quite stable. Sulfhydryl-containing proteins or other molecules modified with this reagent may be used in photoaffinity labeling studies to investigate the specific interactions between two molecules. After mixing a modified protein with a sample, the solution may be photolyzed to create a covalent crosslink between any interacting species. UV photolysis of the benzophenone group results in a highly reactive triplet-state intermediate which can rapidly insert or add to organic components within van der Waals distance (Figure 5.34). Decay of the active-state intermediate returns the photosensitive group to its original chemical form, thus allowing repeated photoactivations without losing the potential for coupling to its intended target.



Benzophenone-4-maleimide
MW 277.27

Benzophenone-4-maleimide is water-insoluble and should be pre-dissolved in DMF or another organic solvent prior to adding an aliquot to an aqueous reaction mixture. Stock solutions may be prepared and stored successfully if protected from light. The hydrophobicity and bulkiness of the benzophenone group may cause insolubility problems in the initial protein that is modified if the derivatization is done at too high a level. Fortunately, the use of a sulfhydryl-reactive reagent can limit the degree of derivatization, since thiol groups usually are present in lower quantities and in more discrete locations than amines.

5. Carbonyl-Reactive and Photoreactive Crosslinkers

Crosslinking reagents containing a photoreactive group on one end and a carbonyl-reactive group on the other end are rare. The use of an amine group on one end of a photosensitive heterobifunctional reagent has been described (Drafler and Marinetti, 1977; Das and Fox, 1979; Gorman and Folk, 1980), but the presence of a hydrazide is required for spontaneous reactivity toward carbonyls. The following compound is the only commercially available reagent containing a phenyl azide photoreactive group and a hydrazide functional group.

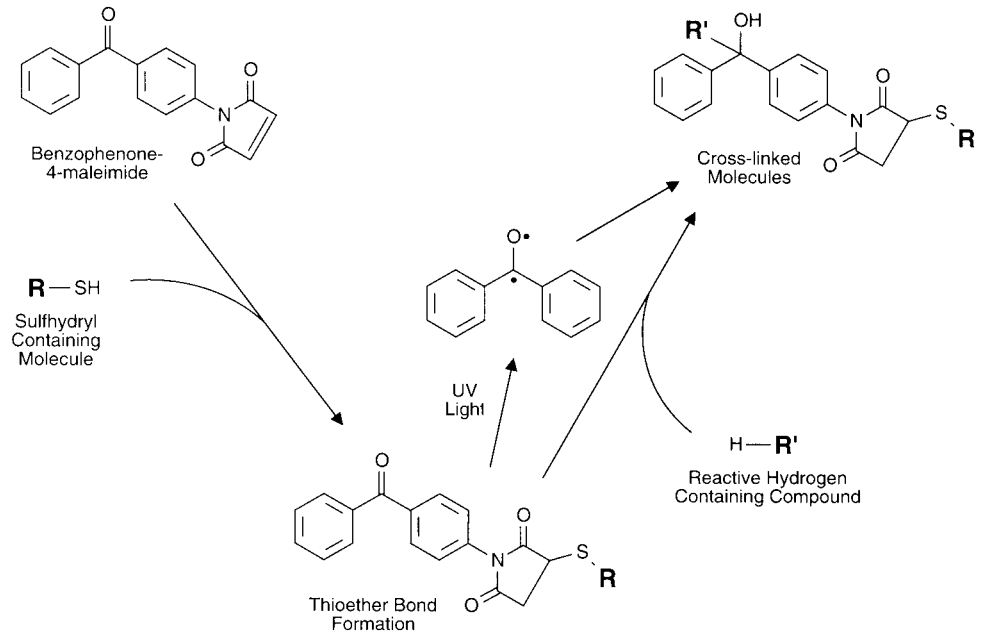
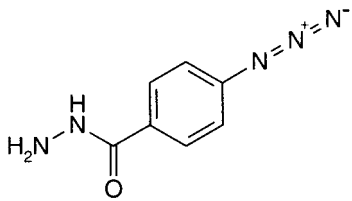


Figure 5.34 Benzophenone-4-maleimide can couple to thiol-containing molecules to form stable thioether bonds. Exposure of the benzophenone group to UV light causes transition to a triplet-state ketone of high reactivity for insertion into C—H or N—H bonds.

5.1. ABH

ABH, *p*-azidobenzoyl hydrazide, is a small, hetero bifunctional crosslinker containing a photoreactive phenyl azide group on one end and a hydrazide functionality on the other end (Thermo Fisher). The hydrazide can react with carbohydrate containing molecules after oxidation with sodium periodate (Chapter 1, Section 4.4) to create aldehyde residues. The reaction forms a hydrazone linkage. Thus, glycoproteins may be specifically labeled on their polysaccharide chains for subsequent investigation of their interaction with receptor molecules (Figure 5.35). In this sense, lectin–carbohydrate interactions may be studied through direct modification of the sugar groups at or adjacent to the binding site. Other amine- or sulfhydryl-reactive probes may not be suitable for such studies due to the lack of amine or sulfhydryl groups near enough to a polysaccharide structure.



ABH
p-Azidobenzoyl hydrazide
MW 177.17
11.9 Å

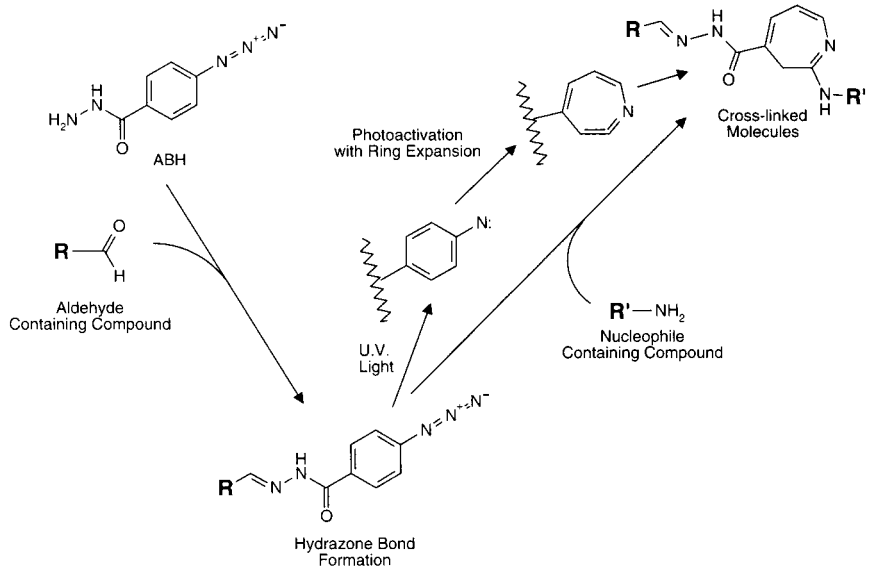


Figure 5.35 ABH reacts with aldehyde-containing compounds through its hydrazide end to form hydrazone linkages. Glycoconjugates may be labeled by this reaction after oxidation with sodium periodate to form aldehyde groups. Subsequent photoactivation with UV light causes transformation of the phenyl azide to a nitrene. The nitrene undergoes rapid ring expansion to a dehydroazepine that can couple to nucleophiles, such as amines.

The cross-bridge of ABH consists of a benzoic acid derivative and thus provides a short spacer between conjugated molecules. After ABH modification of a glycoprotein and incubation with a potential target molecule, the solution may be photolyzed with UV light to initiate the final crosslink. Prior to photolysis, the reagent and all modified species should be protected from light to prevent degradation of the phenyl azide group.

ABH is relatively insoluble when directly added to water or buffer, and therefore it should be pre-dissolved in DMSO prior to addition of an aliquot to an aqueous reaction medium. Stock solutions at a concentration of 50 mM ABH in DMSO work well. Since both reactive groups on ABH are stable in aqueous environments as long as the solution is protected from light, a secondary stock solution may be made from the initial organic preparation by adding an aliquot to the hydrazide reaction buffer (0.1 M sodium acetate, pH 5.5; O'Shannessy *et al.*, 1984; O'Shannessy and Quarles, 1985). Make a 1:10 dilution of the ABH/DMSO solution in the reaction buffer. This solution may be stored in the dark at 4°C without decomposition.

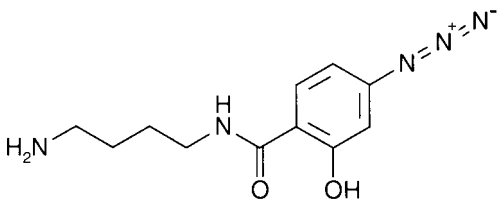
6. Carboxylate-Reactive and Photoreactive Crosslinkers

A carboxylate-reactive crosslinking compound typically contains a primary amine functional group that can be coupled to a carboxylic acid group in a protein or other molecule through the use of a suitable activating agent, such as a carbodiimide. The carbodiimide forms an active ester intermediate that then reacts with the amine to create an amide bond (Chapter 3, Section 1). Reported use of diazoalkyl derivatives that spontaneously react with carboxylates have been tried with fluorescent probes, but not yet applied to heterobifunctional crosslinking agents (DeMar *et al.*, 1992; Schneede and Ueland, 1992) (Chapter 2, Section 3.1). The

following heterobifunctional reagent is the only carboxylate-reactive photosensitive crosslinker currently available commercially.

6.1. ASBA

ASBA, 4-(*p*-azidosalicylamido)butylamine, is a carboxylate-reactive crosslinking agent containing a primary amine on one end and a photosensitive phenyl azide group on the other (Thermo Fisher). The crosslinker is not spontaneously reactive with carboxylates, but must be used with another activating agent that facilitates bond formation. For instance, it can be used in conjunction with a carbodiimide or other such reagent system that can initiate covalent bond formation with a carboxylic acid. A water-soluble carbodiimide-like EDC (Chapter 3, Section 1.1) is able to activate the carboxylates on a target molecule, forming active ester intermediates (Figure 5.36). In the presence of ASBA, derivatization will occur resulting in amide bond formation, and thus leading to modification of the carboxylate-containing molecule with a photoreactive group.



ASBA
4-(*p*-Azidosalicylamido)
butylamine
MW 249.27
16.3 Å

The cross-bridge of ASBA provides a reasonably long spacer (16.3 Å). The phenyl azide portion is constructed from a salicylic acid derivative and thus possesses a ring-activating hydroxyl group. The presence of this group allows radioiodination of the ring prior to crosslinking (Chapter 12, Section 5). Before the photolyzing step is initiated, the reagent should be handled in the dark or protected from light to avoid decomposition of the phenyl azide group.

ASBA has been used to identify parasite adhesive proteins (Gowda *et al.*, 2007), for active site-directed labeling of glucosidase I (Romaniouk *et al.*, 2004), and to study interactions with the proteasome (Qureshi *et al.*, 2003).

7. Arginine-Reactive and Photoreactive Crosslinkers

The guanidinyl group on arginine's side chain can be specifically targeted by the use of 1,2-dicarbonyl reagents, such as the diketone group of glyoxal (Chapter 2, Section 5.2). Under alkaline conditions, this type of group can condense with the guanidinyl residue to form a Schiff base-like complex. The presence of other chemical compounds in the reaction can cause further structural rearrangements, such as stabilization by boronate (Pathy and Smith, 1975). Derivatives such as phenylglyoxal and *p*-nitrophenylglyoxal can be used to block or quantitatively determine the amount of arginine in a protein (Yamasaki *et al.*, 1981). Studies have shown that if the reaction is done with a 2:1 ratio of glyoxal compound to arginine residues,

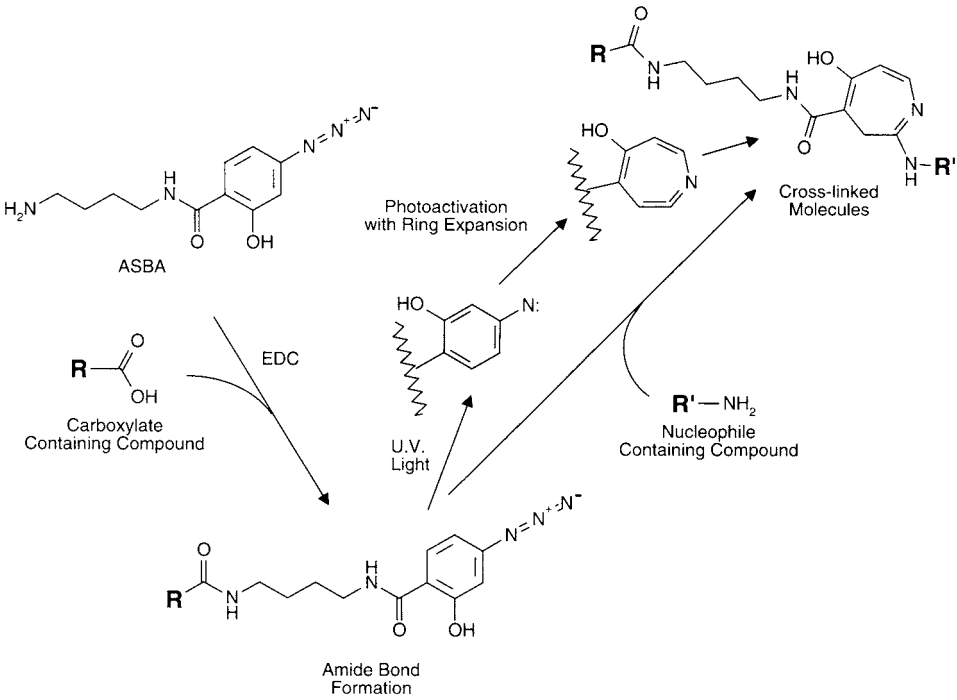


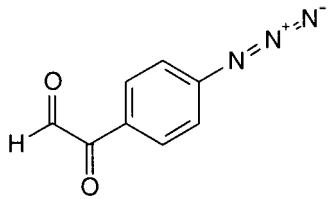
Figure 5.36 ASBA contains a primary amine group that may be conjugated to carboxylate compounds using the carbodiimide EDC. Subsequent exposure to UV light initiates the photoreaction leading to covalent crosslinks.

then the modification that results is reversible (Takahashi, 1968). However, if the modification is done at a 1:1 stoichiometry, then it is irreversible (Konishi and Fujioka, 1987).

The ability to direct conjugation or modification specifically through arginine residues using this chemistry has been exploited in the availability of the only photoreactive glyoxal derivative, APG.

7.1. APG

APG, *p*-azidophenyl glyoxal, is a heterobifunctional crosslinker containing an arginine-specific diketone group on one end and a photosensitive phenyl azide group on the other end (Thermo



APG
p-Azidophenyl glyoxal
MW 193.16
(as the monohydrate)
9.3 Å

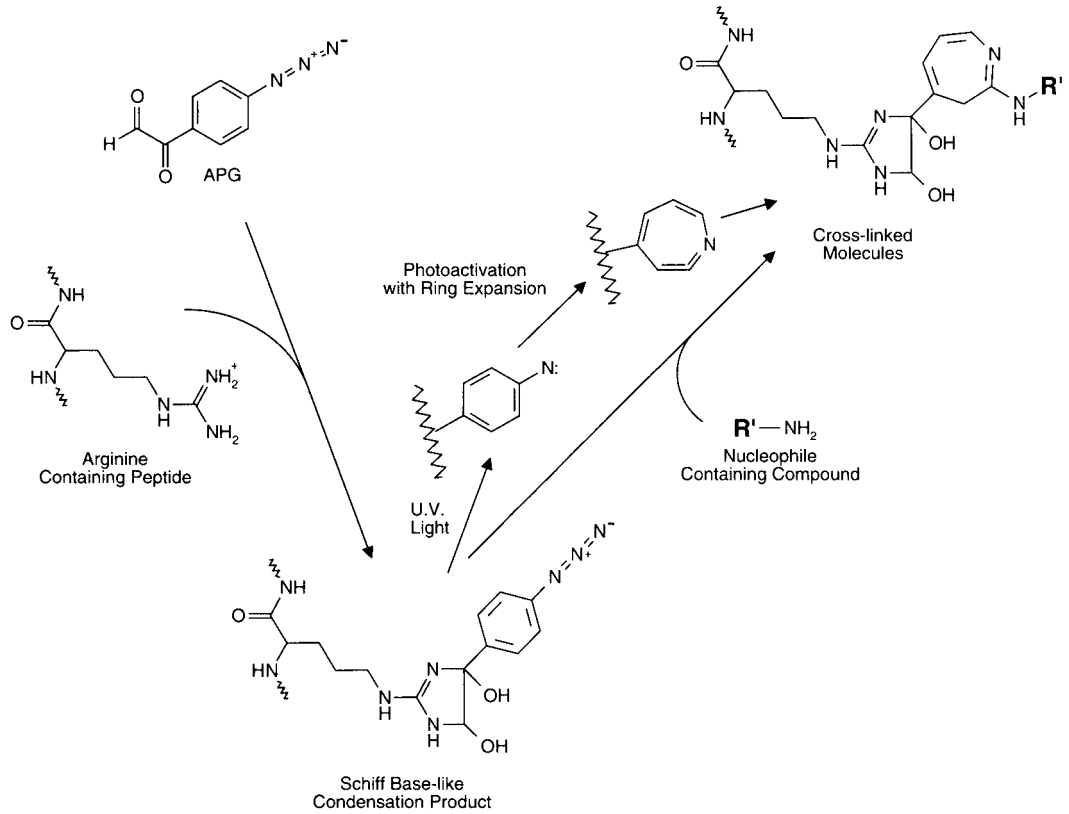


Figure 5.37 APG can be used to label specifically arginine residues in proteins, producing stable, cyclic Schiff base-like bonds with the side-chain guanidino groups. Photoactivation with UV light then causes ring expansion of the phenyl azide group, initiating covalent bond formation with amines.

Fisher). The reagent is a derivative of phenylglyoxal, a compound long used as an arginine guanidinyl modifier. Reaction of APG with proteins at pH 7–8 results in selective modification of arginine, leaving photoreactive groups available for subsequent crosslinking with interacting molecules (Figure 5.37). Exposure to UV light effects the final crosslink. The cross-bridge of an APG crosslink is only 9.3 Å in length, allowing proximity interactions to be studied or the irreversible labeling of arginine areas in proteins.

APG has been used to investigate the binding step in collagen phagocytosis (Chong *et al.*, 2007), to inhibit bovine heart lactic dehydrogenase, egg white lysozyme, horse liver alcohol dehydrogenase, and yeast alcohol dehydrogenase (Ngo *et al.*, 1981), crosslinking RNA–protein interactions in *E. coli* ribosomes (Politz *et al.*, 1981), and identifying regions of brome mosaic virus coat protein chemically crosslinked *in situ* to viral RNA (Sgro *et al.*, 1986).

6

Trifunctional Crosslinkers

Trifunctional crosslinkers represent a relatively small but important category of bioconjugation reagents, possessing three different reactive or complexing groups per molecule. The design of this type of reagent is more elaborate than multifunctional crosslinkers such as polyaldehyde dextran (Chapter 25, Section 2.1) or small organic molecules like trichloro-*s*-triazine (Chapter 25, Section 1.1) which merely contain more than two groups of the same functionaity per molecule. The trifunctional approach incorporates elements of the heterobifunctional concept wherein two ends of the linker contain reactive groups able to couple with two different functional groups on target molecules. A trifunctional reagent, however, has a third arm terminating in still another group able to specifically link to a third chemical or biological target.

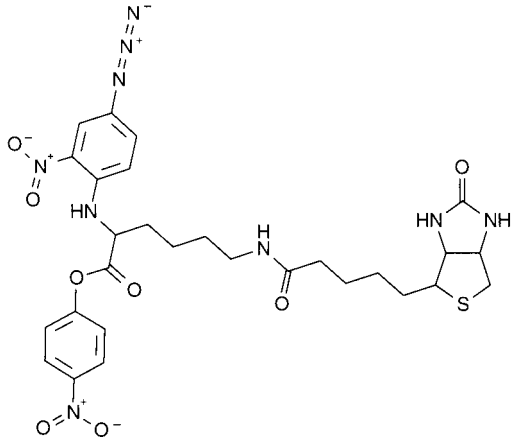
A convenient molecule from which to build trifunctionals is the amino acid, L-lysine. Its three functional groups, α-carboxy, α-amino, and ε-amino, can be derivatized independently to contain three arms. Each arm can be designed to terminate in a complexing group able to participate in a particular type of conjugation reaction or affinity interaction.

The initial attempts at producing trifunctional reagents used biocytin as the core compound. Biocytin is the lysine derivative of biotin having its valeric acid side chain amide-bonded to the ε-amino group of the amino acid (Chapter 11, Section 3). Thus, crosslinkers built on this compound have one of their trifunctional arms ending in a biotin label which is able to specifically complex with avidin or streptavidin probes. Creating two additional reactive arms from the α-carboxy and α-amino groups of biocytin results in the completed trifunctional. The following two crosslinkers are examples of this approach.

1. 4-Azido-2-nitrophenylbiocytin-4-nitrophenyl ester

Wedekind *et al.* (1989) designed a trifunctional reagent for studying the hormone binding site of the insulin receptor. The crosslinker, 4-azido-2-nitrophenylbiocytin-4-nitrophenyl ester (ABNP) contains a nitrophenyl ester group that can react with amine functions in proteins and peptides, similar to the reaction of *N*-hydroxysuccinimide (NHS) esters with amines (Chapter 2, Section 1.4). This group can be used to modify a ligand (such as insulin) prior to its binding to a specific receptor molecule. The second chemically reactive functional group on ABNP is a photosensitive pheny lazide group capable of being activated by exposure to UV light. After

the labeled ligand is allowed to interact with its receptor, forming an interaction complex, the mixture is photolyzed to effect a covalent attachment point (Figure 6.1). The third arm of the trifunctional reagent is the biotin handle (from biocytin). This component allows the complex to be purified by affinity chromatography on immobilized avidin or immobilized streptavidin. Alternatively, the biotin group can be used to visualize the binding of the ligand to its receptor using labeled avidin or streptavidin reagents (Chapter 23).

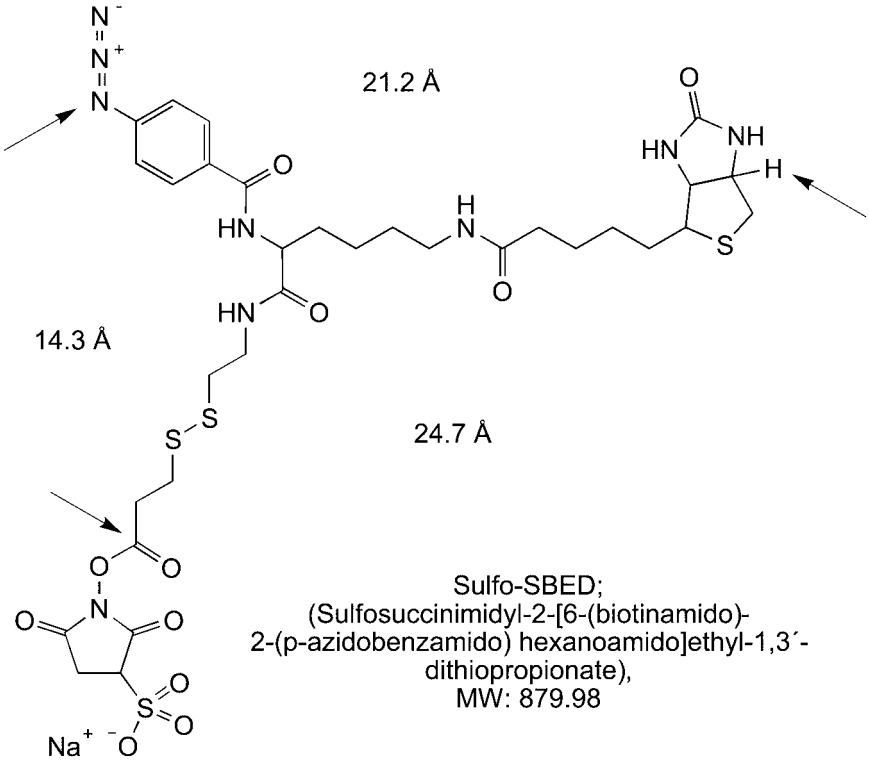


4-Azido-2-nitrophenylbiocytin-4-nitrophenyl ester

ABNP is soluble in dimethylformamide (DMF) but insoluble directly in aqueous solution. Insulin labeling was done in DMF:water at a ratio of 9:1. For molecules not soluble in organic solvent, such as proteins, the trifunctional first may be dissolved in DMF and a small aliquot added to an aqueous reaction medium. The nitrophenyl ester reactive group can be coupled to amine groups at alkaline pH (7–9) and in buffers containing no extraneous amines (avoid Tris). Unfortunately, ABNP is not commercially available at the time of this writing.

2. Sulfo-SBED

Another trifunctional crosslinking agent is sulfo-SBED or sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)hexanoamido]ethyl-1,39-dithiopropionate, developed by Ed Fujimoto at Pierce Chemical (now Thermo Fisher). Like ABNP discussed previously, sulfo-SBED is built on a biocytin backbone. Thus, one arm of the trifunctional compound consists of a biotin handle that can be used for purification or detection purposes using avidin or streptavidin probes. The chemically reactive groups of sulfo-SBED include a sulfo-NHS ester and a phenyl azide group. The sulfo-NHS ester provides amine-coupling capability, forming amide bond linkages with target molecules (Chapter 2, Section 1.4). The phenyl azide is photosensitive and may be activated by exposure to UV light at wavelengths >300 nm. Most phenyl azides react by ring expansion to dehydroazepines with subsequent reactivity toward nucleophiles, especially amines (Chapter 2, Section 7.1) (Figure 6.2).



Sulfo-SBED;
(Sulfosuccinimidyl-2-[6-(biotinamido)-
2-(p-azidobenzamido) hexanoamido]ethyl-1,3´-
dithiopropionate),
MW: 879.98

The sulfo-NHS ester of sulfo-SBED is negatively charged and provides a degree of water solubility (about 5 mM maximum concentration) for the entire molecule. Limited water solubility is all that can be expected due to the large size of the trifunctional, most of it consisting of relatively hydrophobic structures. However, the reagent is much more soluble in organic solvents such as DMF (170 mM) and dimethyl sulfoxide (DMSO) (125 mM). Concentrated stock solutions may be prepared in these solvents prior to addition of a small aliquot to an aqueous reaction mixture.

Since the active ester end of the molecule is subject to hydrolysis (half-life of about 20 minutes in phosphate buffer at room temperature conditions), it should be coupled to an amine-containing protein or other molecule before the photolysis reaction is done. During the initial coupling procedure, the solutions should be protected from light to avoid decomposition of the phenyl azide group. The degree of derivatization should be limited to no more than a 5- to 20-fold molar excess of sulfo-SBED over the quantity of protein present to prevent possible precipitation of the modified molecules. For a particular protein, studies may have to be done to determine the optimal level of modification.

An additional feature of sulfo-SBED is the presence of a cleavable disulfide group in the cross-bridge of the NHS ester arm of the molecule. After a conjugation reaction has taken place, the complexes first may be purified using immobilized avidin or immobilized streptavidin and then the conjugates released by treatment with a disulfide reducing agent. This allows analysis of the complexed molecules, for example, after the binding of a ligand to its receptor. Alternatively, the disulfide group may be cleaved after interaction and capture of unknown

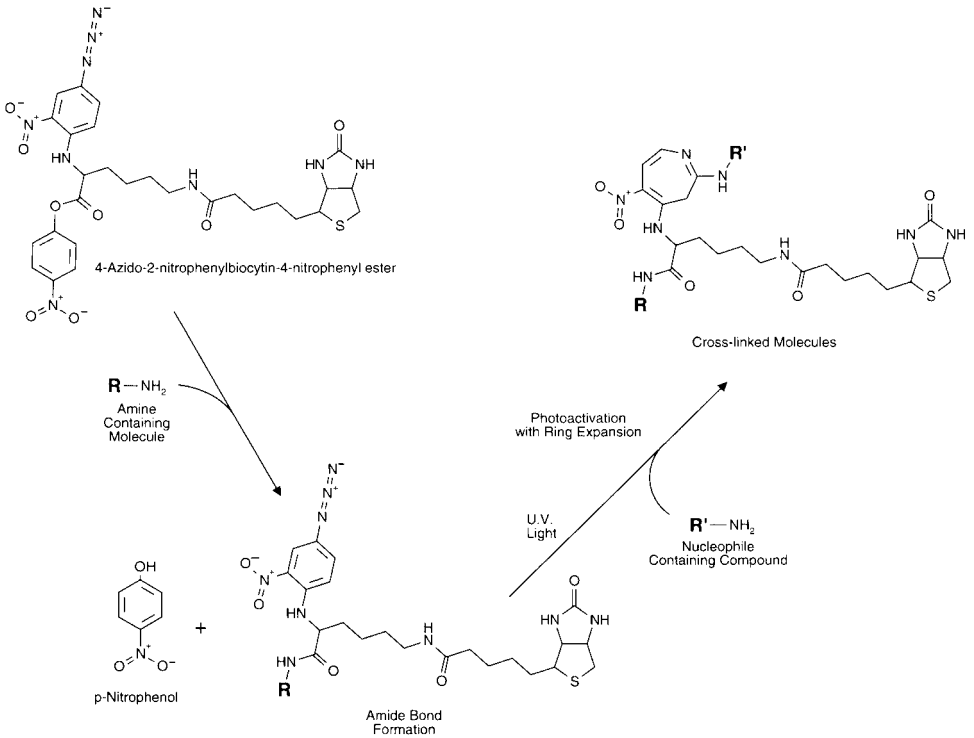


Figure 6.1 The Wedekind trifunctional crosslinker can react with amine groups via its *p*-nitrophenyl ester to form amide bond linkages. The phenyl azide group then can be photoactivated with UV light to generate covalent bond formation with a second molecule. The biotin side chain provides binding capability with avidin or streptavidin probes.

proteins, thus transferring the biotin label to the prey protein. The unknown interacting protein then may be detected or purified using the biotin tag. Such label transfer procedures are important options for studying protein–protein interactions (Chapter 28).

Since sulfo-SBED has three functional arms, the length of each portion should be considered when doing conjugation studies involving interacting proteins. The biotin handle has an effective length of 19.1 Å, including the side chain length for the lysine component. The sulfo-NHS ester arm is approximately 13.7 Å long, measuring from the same point in the lysine group. The phenyl azide arm is the shortest, only 9.1 Å long. The structure for sulfo-SBED shown in this section includes molecular distance measurements somewhat different from these numbers in that the total distances between the three arms are given, which reflects the intramolecular distances between the terminal reactive groups or interacting group on biotin (indicated by the arrows).

Additional information on the use of sulfo-SBED for the study of protein interactions can be found in Chapter 28, Section 3.1.

The following suggested protocol was developed by Barb Olson at Thermo Fisher for the labeling of soybean trypsin inhibitor (STI) with its subsequent complexation with trypsin. Modifications to this procedure may have to be done for other proteins.

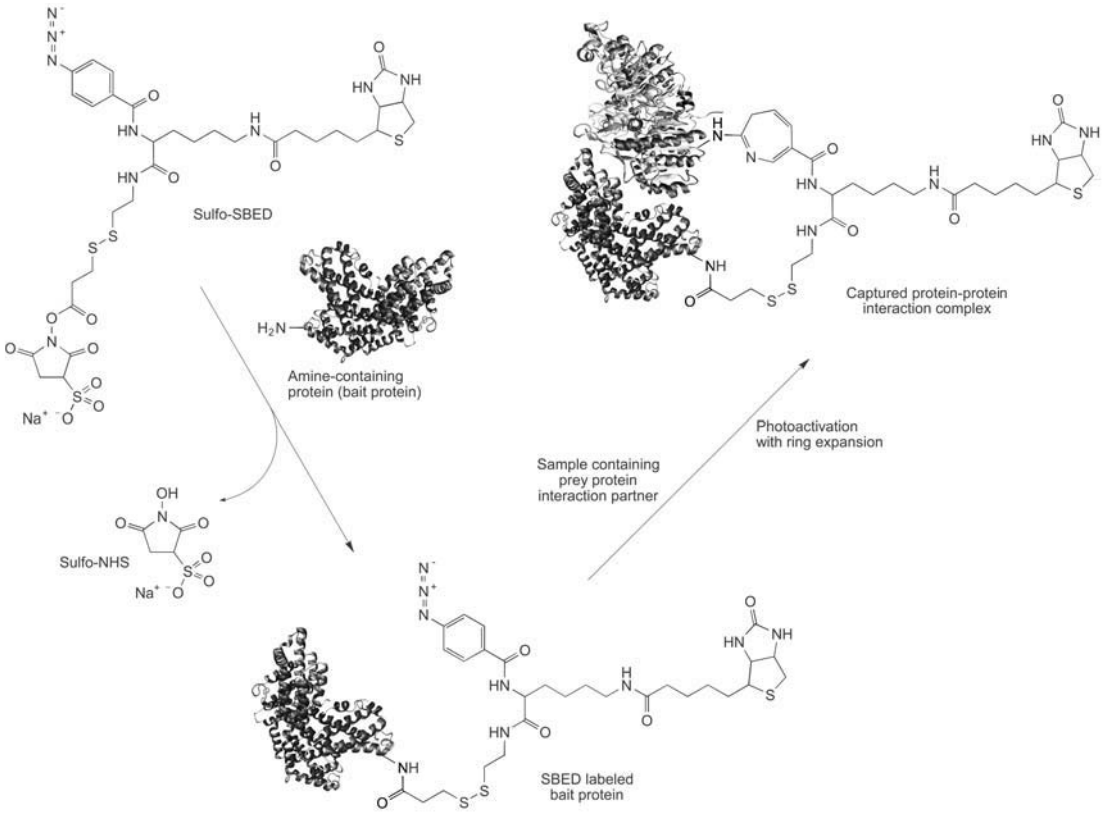


Figure 6.2 The trifunctional reagent sulfo-SBED reacts with amine-containing bait proteins via its NHS ester side chain. Subsequent interaction with a protein sample and exposure to UV light can cause crosslink formation with a second interacting protein. The biotin portion provides purification or labeling capability using avidin or streptavidin reagents. The disulfide bond on the NHS ester arm provides cleavability using disulfide reductants, which effectively transfers the biotin label to an unknown interacting protein.

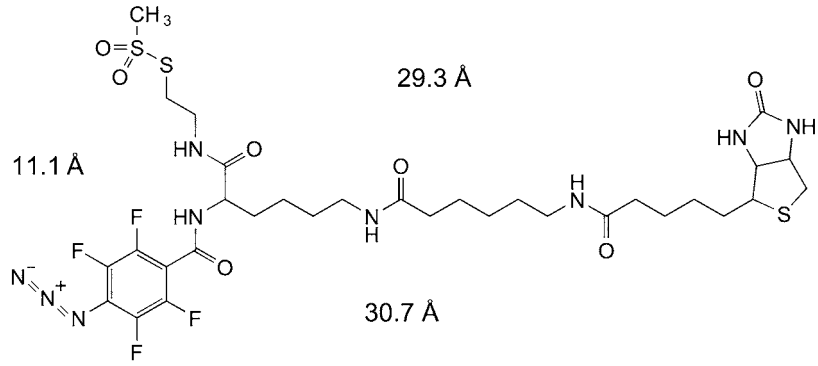
Protocol

1. Dissolve 5 mg of STI in 0.5 ml 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. In a fume hood, dissolve 1.12 mg of sulfo-SBED in 25 µl of DMSO. Prepare fresh and protect from light.
3. Add 11 µl of the sulfo-SBED solution to the STI solution. Mix well.
4. React for 30 minutes at room temperature or for 2 hours at 4°C.
5. If some precipitation occurs, clarify the solution by centrifugation using a microfuge. Remove excess reactant by gel filtration using a desalting resin.
6. Mix the purified sulfo-SBED-modified STI with 5 mg of trypsin dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
7. Incubate at room temperature 3.5 minutes to allow the specific binding of the two molecules to occur.
8. Photolyze the solution with long UV light (about 365 nm) at a distance of about 5 cm for 15 minutes. This process may be done with the solution on ice to prevent heating of the sample.

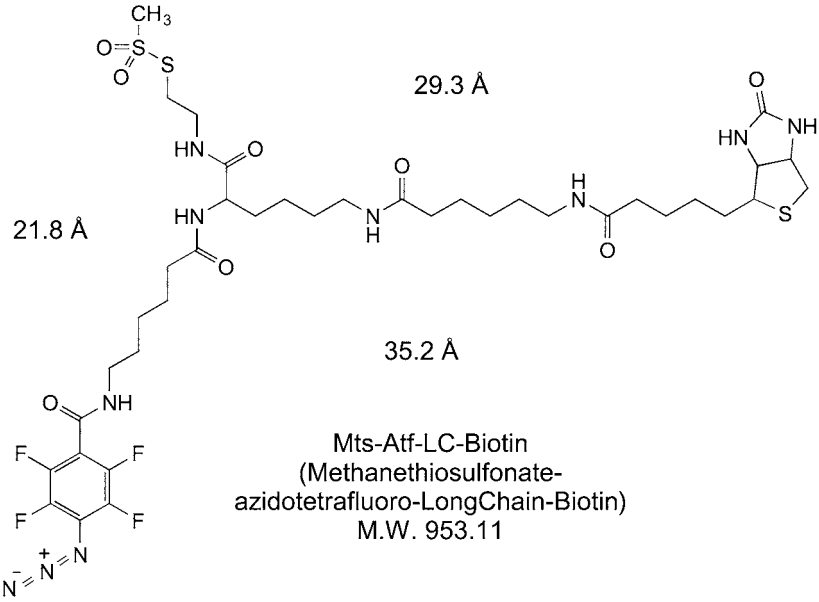
Isolation of complexed molecules may be done by affinity chromatography using a column of immobilized avidin or immobilized streptavidin. Cleavage of the disulfide bond of the crosslinker may be done by treatment with 50 mM dithiothreitol (DTT). For additional information on the use of sulfo-SBED in the study of protein interactions, see Chapter 28, Section 3.1.

3. MTS-ATF-Biotin and MTS-ATF-LC-Biotin

MTS-ATF-biotin and MTS-ATF-LC-biotin are trifunctional crosslinkers similar in design to sulfo-SBED discussed previously, but in addition to the biotin handle, they contain a



Mts-Atf-Biotin
(Methanethiosulfonate-
azidotetrafluoro-Biotin)
M.W. 839.95



Mts-Atf-LC-Biotin
(Methanethiosulfonate-
azidotetrafluoro-LongChain-Biotin)
M.W. 953.11

thiol-reactive group and an enhanced photoreactive, perfluorinated phenyl azide group. The two reagents differ only in the length of the cross-bridge in the photoreactive arm, with the LC version containing an extended aminocaproyl spacer. Relative to the spacing possible between the reactive groups on these compounds, the LC version therefore provides nearly twice the maximal molecular distance over its shorter analog (21.8 Å versus 11.1 Å). Thus, interacting proteins may be captured either through use of a long or short crosslink, depending on the optimal distances between the proteins—or at least to the nearest thiol on the bait protein.

Both MTS-ATF-biotin and MTS-ATF-LC-biotin contain a methanethiolsulfonate group (MTS) on one arm, which is able to couple with thiols. This reaction proceeds with loss of the methyl sulfonate leaving group (sulfinic acid) and forms a disulfide linkage (Figure 6.3). Unlike a pyridyl disulfide group, however, which also reacts with thiols to form disulfide linkages, the MTS group is unstable to hydrolysis in aqueous solution, especially if other strong nucleophiles are present. Therefore, most MTS compounds dissolved or brought into PBS buffer at physiological pH will hydrolyze with a half-life on the order of 10–15 minutes. However, they also have very rapid reactivity with thiols (Stauffer and Karlin, 1994; Holmgren *et al.*, 1996; Liu *et al.*, 1996). The reaction of an MTS group with a thiol on a bait protein can take place with high yields in just a matter of minutes. Both of these trifunctional label transfer compounds are hydrophobic, so their MTS reactivity in the aqueous phase may be somewhat slower than corresponding hydrophilic MTS reagents.

For use in studying protein interactions, these compounds first are reacted with a thiol on a purified bait protein to form a disulfide bond. Since the reagents are water-insoluble, they must be dissolved in an organic solvent such as DMF or DMSO and then an aliquot added to the bait protein in an aqueous buffer to initiate the reaction. Once modified, the biotinylated bait protein then is incubated with a sample containing potentially interactive prey proteins. After an incubation period, initiating the photoreaction by exposure to UV irradiation captures the interacting proteins. Any interaction complexes thus formed can be isolated or detected using the biotin handle. In addition, the disulfide bond formed with the bait protein during the crosslinking reaction can be reduced to cleave the conjugates and transfer the biotin label to the unknown interacting prey proteins. This is a powerful way of labeling unknown interacting proteins for subsequent analysis.

Although MTS-ATF-biotin and MTS-ATF-LC-biotin are available commercially (Thermo Fisher and Toronto Research), they are relatively new and don't have the publications or applications backing up their use as sulfo-SBED. A protocol for the use of these compounds in the study of protein interactions can be found in Chapter 28, Section 3.2.

4. Hydroxymethyl Phosphine Derivatives

Although phosphine compounds often are used as disulfide reducing agents (Chapter 1, Section 4.1), there are classes of organo-phosphine reagents containing three hydroxymethyl groups that can act as trifunctional bioconjugation agents for coupling or crosslinking purposes. Tris (hydroxymethyl)phosphine (THP) and β-[tris(hydroxymethyl)phosphino] propionic acid (THPP; Thermo Fisher) are small trifunctional compounds that spontaneously react with nucleophiles, such as amines, to form covalent linkages (Henderson *et al.*, 1994; Katti, 1996; Katti *et al.*,

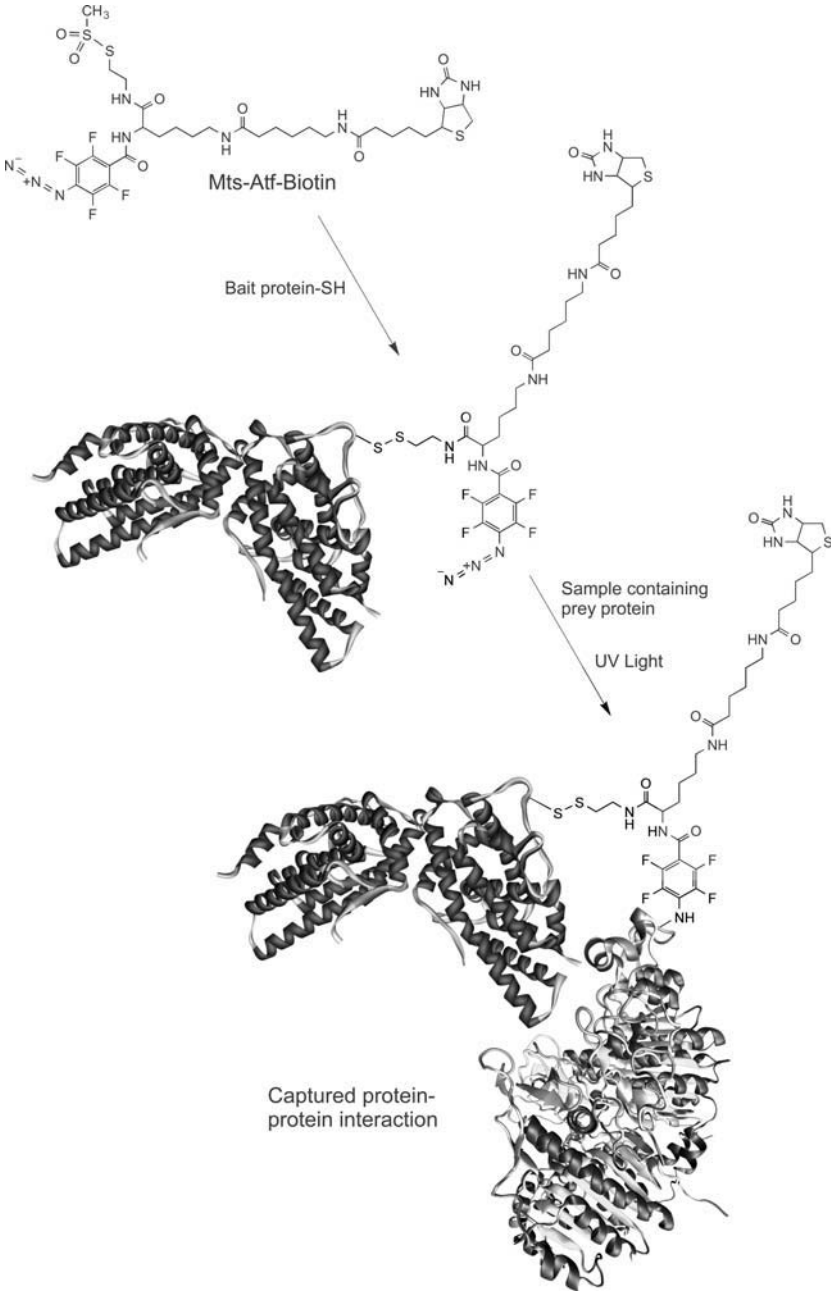
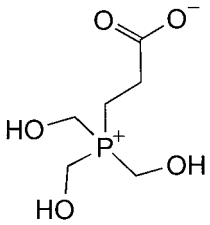
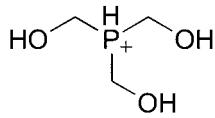


Figure 6.3 Mts-Atf-Biotin can be used to label bait proteins at available thiol groups using the MTS group, which forms a disulfide linkage after reaction. The modified protein then is allowed to interact with a protein sample and photoactivated with UV light to cause a covalent crosslink with any interacting proteins. Cleavage of the disulfide bond effectively transfers the biotin label to the unknown interacting protein.

1999). Nucleophiles react with the hydroxymethyl arms by attack on the electron-deficient carbon atom with loss of water to form secondary or tertiary amine bonds (Figure 6.4).

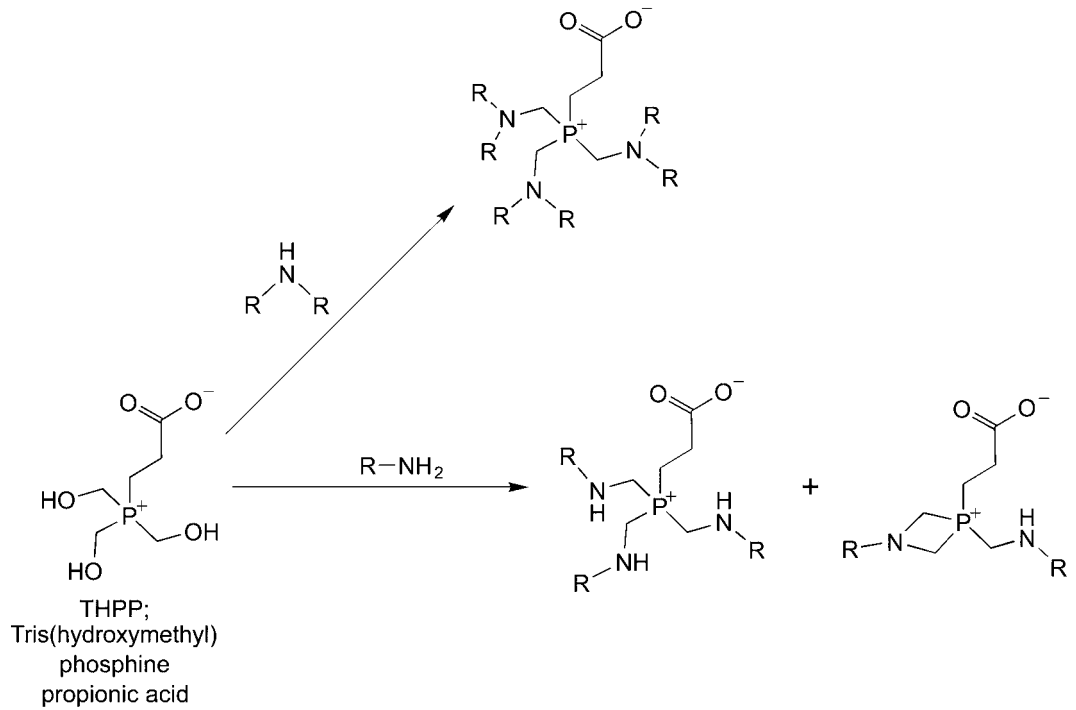


THPP;
Tris(hydroxymethyl)
phosphine propionic acid



THP;
Tris(hydroxymethyl)
phosphine

Both THP and THPP are stable in aqueous solution, as the only potential product of hydrolysis is the reformation of the hydroxymethyl groups. It is unusual for an amine-reactive functional group to have long-term stability in water or buffer, which makes these reagents uniquely suitable for creating reactive surfaces or reactive molecules for subsequent



THPP;
Tris(hydroxymethyl)
phosphine
propionic acid

Figure 6.4 THPP reacts with amine-containing molecules to form secondary or tertiary amine bonds.

conjugation with proteins or other amine-containing compounds. Hydroxylic chromatographic supports also have been activated with hydroxymethyl phosphine derivatives for immobilization of enzymes (Petach *et al.*, 1994).

Hydroxymethyl phosphines are susceptible to oxidation to form the phosphine oxide. Therefore, avoid excess oxygen, oxidizing agents, or azide compounds, which react with phosphines in the Staudinger reaction (Chapter 17, Section 5). In addition, metallic surfaces can be modified via the phosphine group to result in hydroxymethyl group substitutions.

7

Dendrimers and Dendrons

1. Dendrimer Construction

The science of nanotechnology has employed many different constructs having low nanometer dimensions, including inorganic scaffolds, biological macromolecules, and various forms of polymers and particles. One of the most defined nanoparticle constructs is a unique polymeric assemblage called a dendrimer. First described in 1983 by Tomalia and Dewald in the application to U.S. patent 4,507,466 and again in 1985 by Tomalia *et al.* and Newkome *et al.*, dendrimers are monodisperse, globular macromolecules grown by successive synthetic steps from a central core molecule. Each step, called a generation, adds a distinct layer to the previous one so that a dendrimer grows out from the core like the branches of a tree. In fact, the name dendrimer comes from the Greek word for tree, or "dendron". The result is a polymeric molecule having a fractal dimensional quality with properties and shape that is determined by the types of monomers used to grow the branches.

Each step in dendrimer synthesis occurs independent of the other steps; therefore, a dendrimer can take on the characteristics defined by the chemical properties of the monomers used to construct it. Dendrimers thus can have almost limitless properties depending on the methods and materials used for their synthesis. Characteristics can include hydrophilic or hydrophobic regions, the presence of functional groups or reactive groups, metal chelating properties, core/shell dissimilarity, electrical conductivity, hemispherical divergence, biospecific affinity, photoactivity, or the dendrimers can be selectively cleavable at particular points within their structure.

Dendrimers have been used for many diverse applications within the biological, chemical, polymer, and nanotechnology fields. Some major applications include their use as multivalent bioconjugation scaffolds, for enhancement of signals in assays, to solubilize hydrophobic molecules in aqueous environments by internal entrapment, to functionalize surfaces and particles for conjugation, as transfection agents for cells, to create targeted therapeutic constructs for the treatment of disease, as carriers of affinity ligands, and as additives for other polymer mixtures. For excellent reviews of dendrimer technology, see Fréchet and Tomalia, 2002, as well as Boas *et al.*, 2006.

The synthesis and structure of a dendrimer can be illustrated by the well-known poly (amidoamine) type (called PAMAM), which describes the monomers making up the complete polymer. The synthesis starts from a core diamine (or ammonia) molecule. The diamine can be of various lengths and spacer arm properties and even contain cleavable components. Typically, the core

is a short diamine, such as ethylenediamine. The first reaction that is done to form a dendrimer is reacting the core with methyl acrylate to form the Michael addition product, a tetra-methyl ester branched molecule. Next ethylenediamine is again added in large excess to the tetra-methyl ester intermediate, which undergoes amidation to form the tetra-amidoethylamine generation-0 (G-0) product containing 4 pendent amines. Another round of methyl acrylate addition followed by ethylenediamine yields the 8-amine generation 1 (G-1) PAMAM dendrimer (Figure 7.1).

Similar successive additions of methyl acrylate and ethylenediamine result in progressively higher generation dendrimers, which branch out to larger diameters and contain greater numbers of amines on their surface. For dendrimers of the classic PAMAM type, each additional generation results in doubling the number of pendent amine groups decorating its surface, because alkylation of each terminal amine can be done twice with two molecules of methyl acrylate. Since each step in dendrimer synthesis adds greater branching of monomer units as they grow out from the core, the design has become known as "starburst" dendrimers. Figure 7.2 illustrates graphically how the growth of dendrimers built from a bifunctional core results in ever more branching as the generational size increases. The corresponding G-3 and G-4 PAMAM dendrimer chemical structures are shown in Figures 7.3 and 7.4. Although a two-dimensional depiction of this dendrimer structure may look like the molecule has nearly perfect circular symmetry, its true three-dimensional structure actually appears more asymmetrical for lower generation dendrimers and like a complex globular protein (Figure 7.5) for generations above G-4.

There are two main methods of synthesizing dendrimers: (1) the divergent method, which involves building the core outward in successive steps or generations as described above for PAMAM dendrimers and (2) the convergent method (Hawker and Fréchet, 1990; Hodge, 1993; Grayson and Fréchet, 2001), which consists of building a single branched tree as it would grow out from the core in the divergent method, but in this case, after synthesis of individual trees, they are linked to the core structure as single units (Figure 7.6). Thus, convergent dendrimers are constructed from the outside in. One advantage of the convergent synthesis strategy is that different dendritic starting materials (dendrons) can be built and combined to form a segment-block dendrimer or a layer-block dendrimer, which consists of polymers of different types within the same dendrimer structure. The only major disadvantage of using the convergent approach to making dendrimers is that the result is limited to rather small dendritic molecules, because as the size of the building blocks increases steric crowding prevents efficient reaction of all the dendrons with the core.

A third method of constructing dendrimers is through self-assembly of engineered building blocks. When the blocks are put together under the correct conditions in solution, they spontaneously assemble into dendritic structures. For instance, dendrimers have been assembled through use of chelating components, which assemble into dendritic structures upon addition of the appropriate metal ions (Denti *et al*., 1992; Balzani *et al*., 1996; Kawa and Fréchet, 1998). Oligonucleotide dendrimers also have been formed by using intelligently designed sequences that hybridize to other oligos in such a way that dendritic molecules spontaneously are created in solution (Genisphere technology). In addition, dendrimers have been made from dendron trees containing interior groups that can self-assemble through hydrogen bonding with groups on neighboring trees, thus forming the complete dendrimer as the adjacent groups interact at the core (Hudson *et al*., 1997; Percec *et al*., 1998).

Finally, dendrimers have been synthesized using solid phase peptide synthesis resins, wherein the core is linked to the resin and the half-dendrimer (dendron) is built out from it in sequential steps (Marsh *et al*., 1996; Swali *et al*., 1997; Wells *et al*., 1998). The advantage of this method

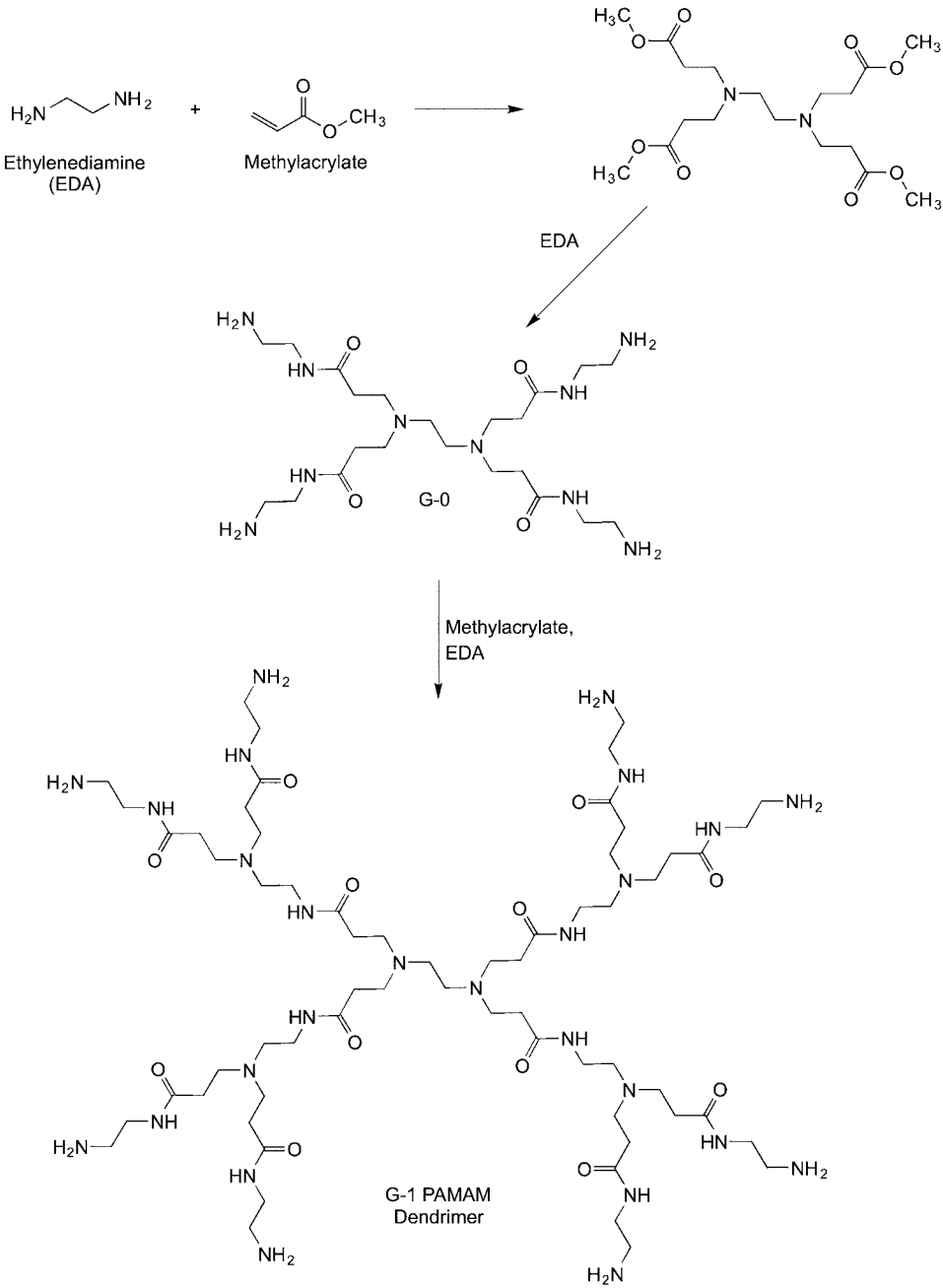


Figure 7.1 The synthesis of a PAMAM-type dendrimer proceeds from a diamine core [e.g., ethylene diamine (EDA)] by initial addition of the amines to the double bonds of methacrylate. Subsequent reaction of the methyl ester groups with EDA produces a G-0 dendrimer with four pendent amine groups. Another round of methacrylate and EDA additions results in a G-1 PAMAM dendrimer containing eight primary amines.

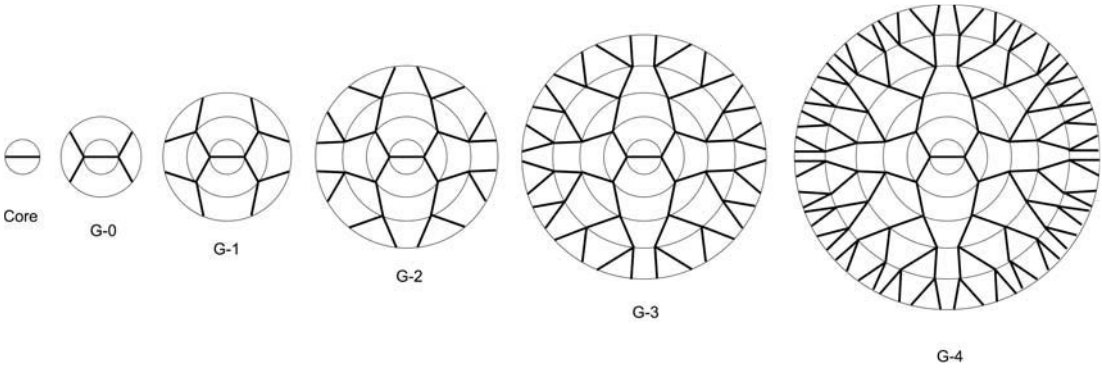
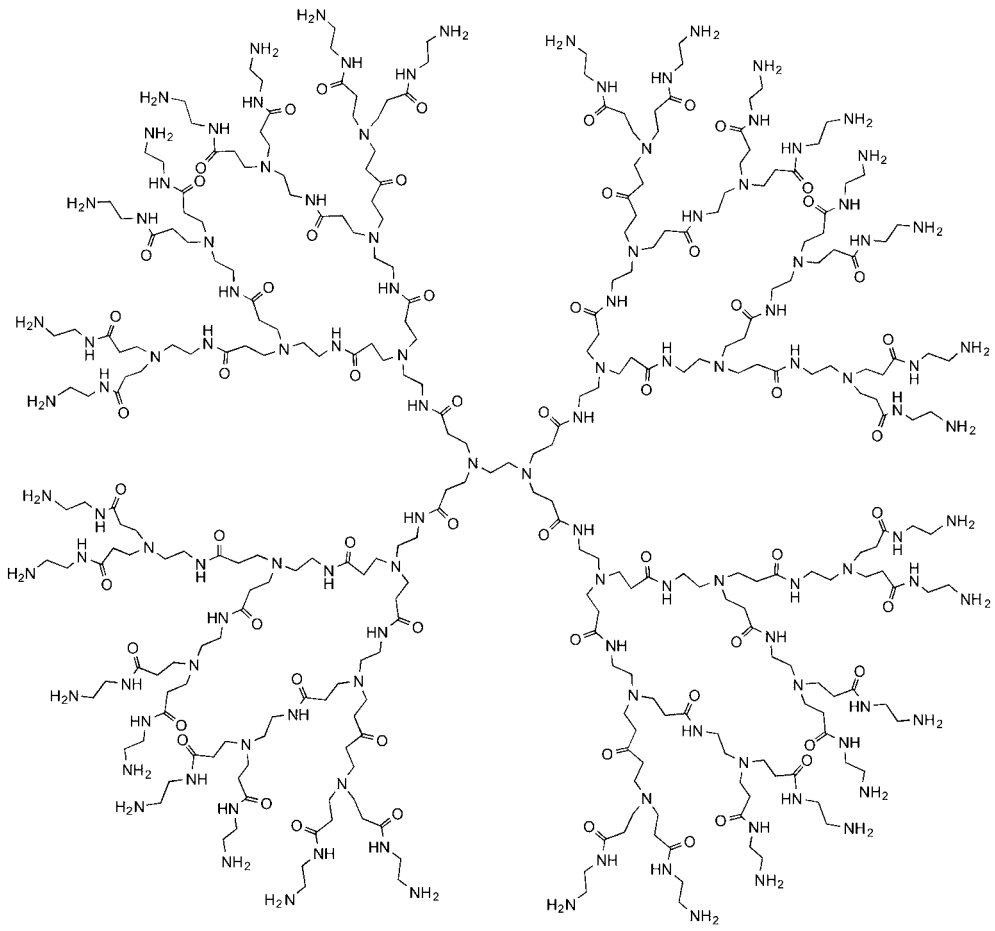


Figure 7.2 A graphical illustration of the growth of dendrimer structures from an initial bifunctional core to a G-4 dendrimer containing 64 terminal groups on its outer surface.



G-3 PAMAM Dendrimer
$C_{302}\ H_{604}\ N_{118}\ O_{60}$
Mol. Wt.: 6848.79

Figure 7.3 The chemical structure of a G-3 PAMAM dendrimer.

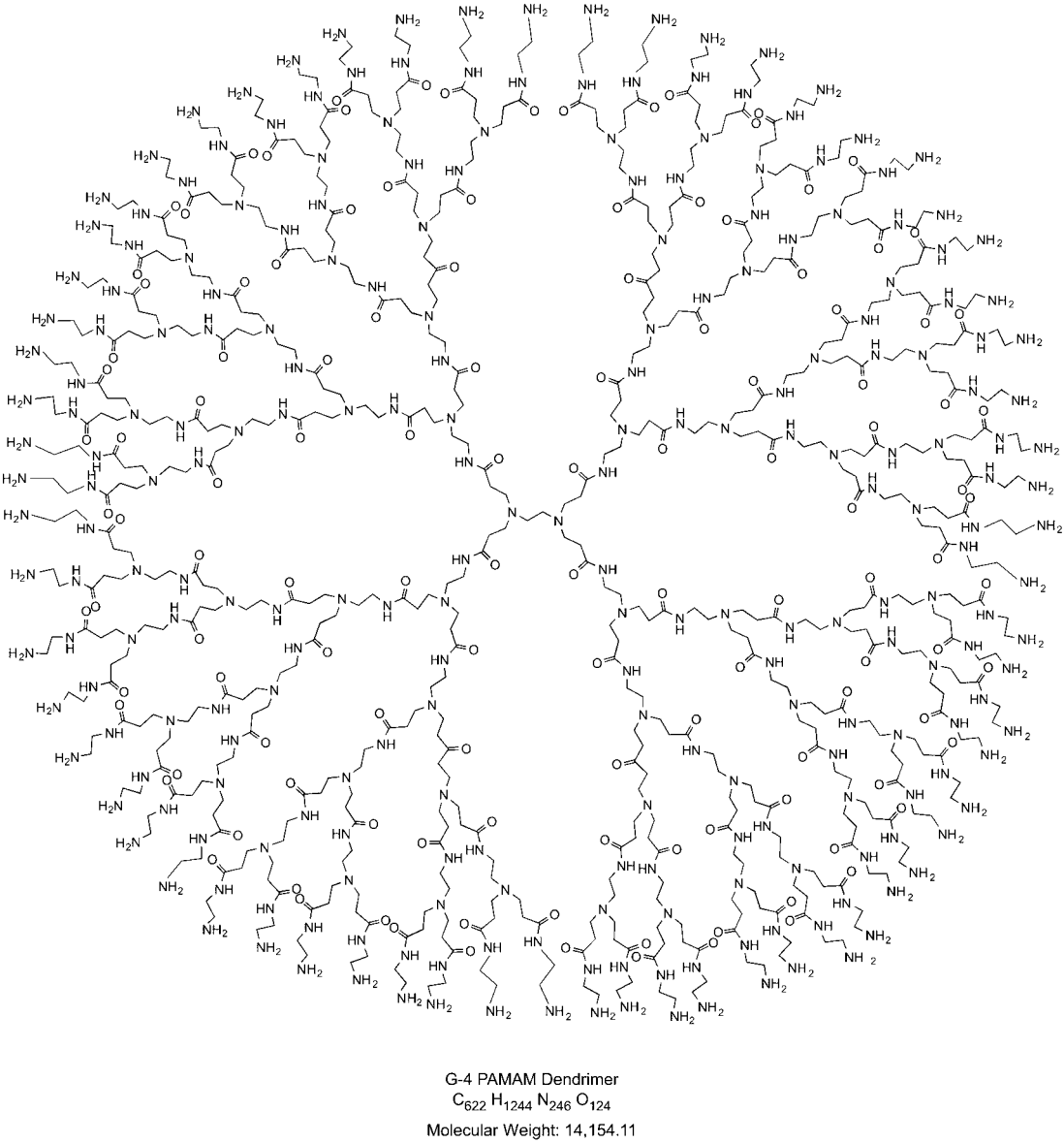


Figure 7.4 The chemical structure of a G-4 PAMAM dendrimer.

is the ease at which reactants are added and the growing dendron is purified from reaction byproducts. This method also can be used to add terminal peptides onto the dendron for affinity targeting purposes. Monaghan *et al.* (2001) used this approach to make peptide–dendrimer conjugates for the investigation of integrin binding.

One of the most important advances in synthesizing dendrimers is through the use of the cycloaddition reaction between azides and alkynes, which has become known as "click

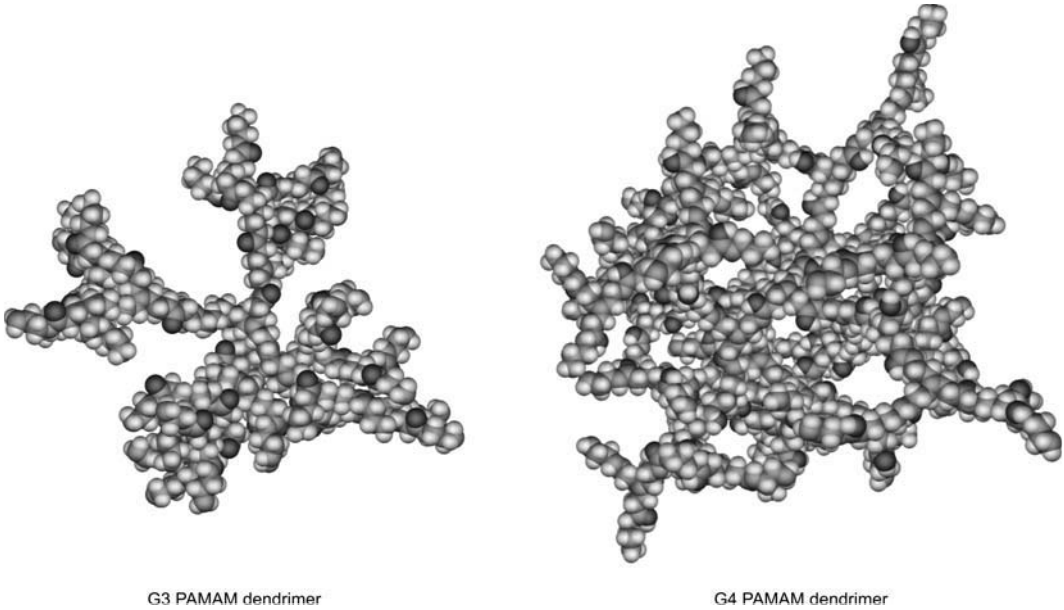


Figure 7.5 The G-3 and G-4 PAMAM structures shown in Figures 7.3 and 7.4 are illustrated here as space-filling molecular models. The G-4 level begins to show similarity to globular proteins in its three-dimensional structure.

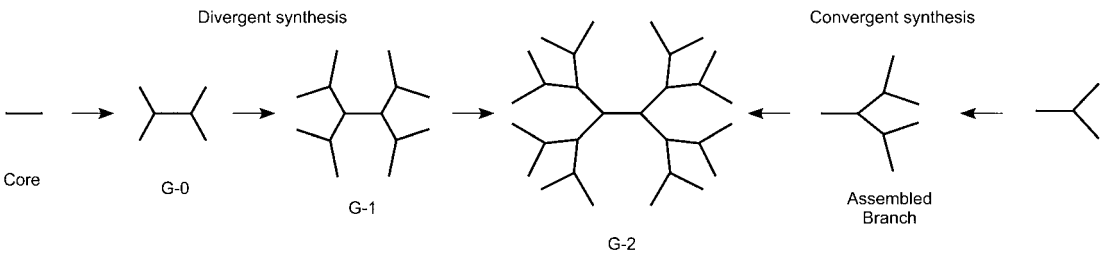


Figure 7.6 The divergent and convergent synthesis methods of dendrimer formation.

chemistry" (Chapter 17, Section 4). The copper I-catalyzed conjugation reaction forms cyclic 1,2,3-triazoles in very high yield. Using the appropriate monomers, the click chemistry-mediated assemblage of dendrimers has provided dramatic improvement to both the yield and the ease of synthesis and thus has dramatically decreased the cost of making bulk amounts of dendritic molecules (Rouhi, 2004; Wu *et al*., 2004, 2005; Joralemon *et al*., 2005; Lee and Kim, 2005) (Figure 7.7). Even unsymmetrical dendrimers can be prepared using a convergent synthesis approach between propargyl-functionalized PAMAM dendrons and azido-functionalized PAMAM dendrons (Lee *et al*., 2007).

Regardless of how they are made, the higher the dendrimer generation the greater the density of its branching becomes. Dendrimers of small size have an internally open configuration that freely permits the flow of small molecules within their inner structure. As dendrimers increase in diameter from G-0 through G-7, their appearance and size becomes more and more similar to

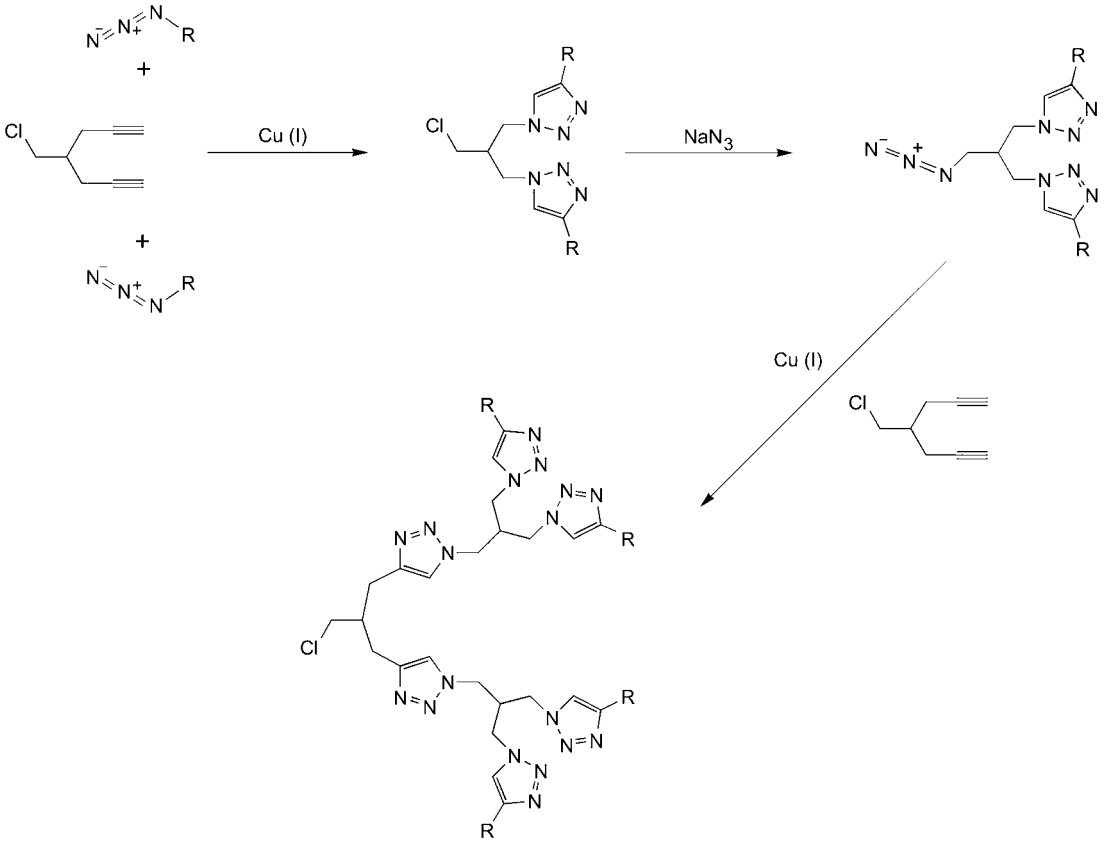


Figure 7.7 The synthesis of dendrimer molecules using click chemistry proceeds with high yield. Each step results in the cycloaddition reaction between azide-containing molecules and alkyne molecules to form triazole linkages.

that of globular proteins of corresponding mass. For instance, a G-0 PAMAM dendrimer has a molecular weight of 517 and a diameter of about 1.5 nm, whereas a G-7 dendrimer has a mass of 116,493 and a diameter of 8.1 nm, which are very similar in mass and diameter to peptides and proteins of comparable sizes (Eichman *et al.*, 2001). Unlike proteins, however, the surface of dendrimers becomes increasingly dense as their size increases. This is due to the doubling of branches and pendent groups on the outer surface for each generation increase in size. As the dendrimer generation and size increases, the molecules become more symmetrical and spherical in shape due to the dense outer branch packing. At a certain point, surface crowding becomes so great that no further access to the internal structure is possible and the dendrimer becomes a rigid ball. As dendrimer size increases, it also becomes increasingly difficult to add another layer of monomers to the surface due to steric hindrance.

In general, dendrimers of size G-0 through G-3 have open, asymmetric, and flexible structures with effectively no protected internal areas, due to a large freedom of motion in their branches, and they can readily accommodate additional covalent attachments to their surfaces. See Figures 7.3–7.5 for illustrations of the two-dimension and three-dimension structure of a

G-3 and G-4 dendrimer, in which G-3 dendrimers can be seen to have a great deal of internal space. However, G-4 through G-6 dendrimers display a more globular structure and contain internal void areas, which can hold guest molecules, such as delivery agents or drugs. Above this size, G-7 and greater, dendrimers are more like solid spheres or particles with inaccessible interiors and highly dense surfaces, and they appear upon imaging much like polymer nanoparticles of similar size (Chapter 14).

Unlike linear or ordinary branched polymers, dendrimers display intrinsically low viscosity, even at high mass. As standard polymeric molecules increase in mass and size, their viscosity normally increases continually. With dendrimers, viscosity increases only up to about the fourth generation, after which it actually begins to decline (Mourey, 1992; Fréchet, 1994). In addition, with control over the type of pendent groups that adorn the surface, dendrimers can maintain high solubility regardless of size.

Dendrimer molecules of the mid-size range have been found to be excellent carriers of guest molecules for solubilization and drug delivery. In addition, certain groups added to the surface of such dendrimers can aid in the entrapment of molecules within the dendrimer cores. Such a construct, called a dendrimer box, can be designed to release the guest molecules upon certain conditions being met, such as pH-facilitated hydrolysis or a photoreaction of the surface capping groups. In this case, the capping groups are reversible and thus the dendrimer can be induced to release its cargo by cleavage (Jansen *et al*., 1994; Jansen and Meijer, 1995). Miklis *et al*. (1997) used molecular dynamics to investigate the encapsulation of rose bengal molecules in a dendrimer box, which was formed by the coupling of tBOC-L-Phe cap molecules to the 64 terminal primary amines of a G-5 poly(propyleneimine) dendrimer. It was discovered that without the capping groups, the rose bengal molecules were in equilibrium between the solvent, surface, and interior of the dendrimer. However, forming a dendrimer box with the tBOC-L-Phe caps stably was found to keep the guest molecules within the dendrimer interior without leakage.

In some cases, a dendrimer box can be designed to provide a slow release of a drug by capping the branched structure with hydrophilic PEG groups. The internal structure is designed to effectively dissolve the organic drug and sequester it, while the PEG capping groups provide extreme dendrimer solubility and inhibit the movement of the drug out of the interior dendritic space. Liu *et al*. (2000) used this design to entrap indomethacin within the hydrophobic branches of a dendrimer built from phenyl-group-containing monomers and provided an $mPEG_{16}$ cap to allow a time-release effect for the drug *in vivo*.

In addition to the PAMAM variety, many different chemical constituents have been used as monomer units to build a dendritic molecule. Some have used trifunctional aromatic units, rigid monomers to keep the branches from bending, polyethers, polyhydroxyls, heterocyclic compounds, etc. In some dendrimer types, the interior structure is hydrophobic and can be used potentially to carry small organic molecules, while the surface groups are made to be hydrophilic to promote overall solubility. The potential variety of dendrimer construction is limited only by the imagination of organic or inorganic building blocks, which can be conceived and linked together.

2. Conjugation to Dendrimers

Many of the applications of dendrimers involve the covalent coupling of other molecules to the dendrimer surface or to points within the branched structure. These attached molecules

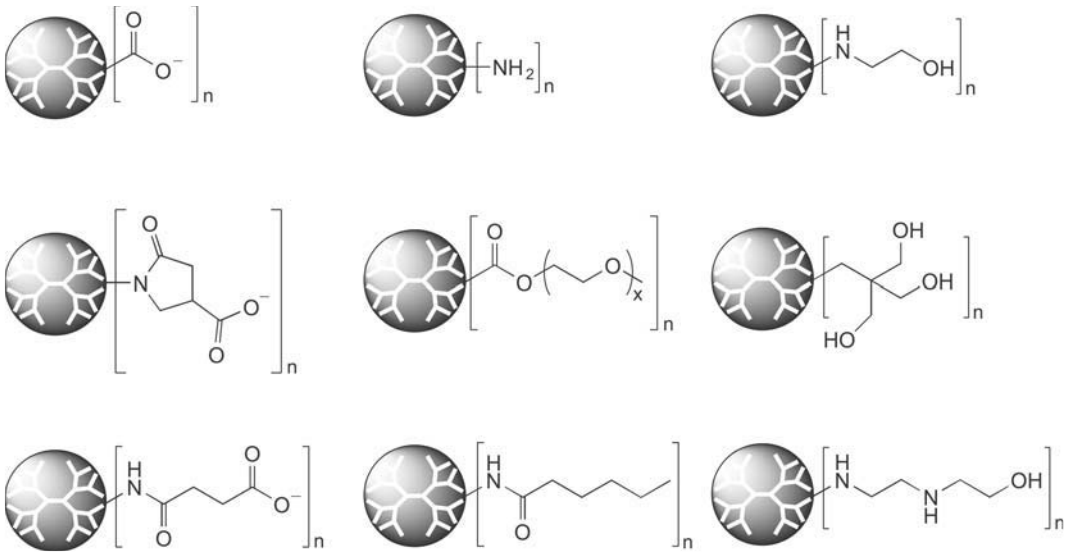


Figure 7.8 The pendent groups available on the surface of dendrimer molecules are highly varied. Some groups provide functional or reactive groups for bioconjugation, while other groups create unique solubility characteristics for the dendrimer.

can function as detection agents, affinity ligands, targeting components, radio-ligands, imaging agents, or pharmaceutically active compounds. The methods used for dendrimer conjugation are similar to the procedures used with other macromolecules and particles. The essential elements in designing a dendrimer conjugate are the functional groups that are present on the dendrimer and the functional groups on the molecule to be coupled.

Dendrimers are commercially available containing a variety of dendron structures and pendent groups on their surface (Dendritic Nanotechnologies; Dendritech). Some of the functionalities available allow for bioconjugation reactions to be done, but others are designed to create certain solubility properties and cannot be used directly for coupling other ligands. Figure 7.8 shows some of the available surface groups, which for traditional PAMAM-based dendrimers, including amine, carboxylate, hydroxyl, methyl ester, mPEG, and a hydrophobic C_6 chain. Selections of different diamine cores also are available with these surface functionalities, including the chain lengths C_2, C_4, C_6, C_{10}, and a disulfide cleavable cystamine core. Most dendrimers of the PAMAM type are commercially available in sizes up to G-6, but can be custom ordered in higher generations, which is more difficult to synthesize and more expensive.

The newer Priostar dendrimers from Dendritic Nanotechnology, which are created using a new manufacturing process, provide greater flexibility in dendrimer design and much better stability in the final product. These dendrimers are stable at room temperature, unlike PAMAM dendrimers, and can withstand extremes in pH or temperature without hydrolyzing or decomposing. In addition, the dendritic structures can be formed to have internal functional groups along their branches for conjugation, including secondary amines and hydroxyls. Therefore, fluorescent molecules or other organic molecules can be attached at internal locations, leaving the surface groups available for additional conjugation sites.

Priostar dendrimers are available with amine, hydroxyl, carboxylate, or epoxy functionalities on their surface for bioconjugation reactions. Also, due to the monomers used for synthesis, each generation of Priostar dendrimer contains more surface functionalities than the corresponding PAMAM dendrimer. For example, PAMAM dendrimers with ethylenediamine cores have 4, 8, 16, and 32 pendent groups present for generations G-0, G-1, G-2, and G-3, respectively. By contrast, Priostar dendrimers have 4, 12, 30, and 100 pendent functionalities on their surface for G-0, G-1, G-2, and G-3, respectively. The result is much greater surface functionality at much lower generation number and size; therefore, surface functionality can be maximized without growing the dendrimer so large that it no longer can accommodate guest molecules within its structure.

Other than the epoxy groups available on one Priostar dendrimer type and a methyl ester available on a PAMAM dendrimer, the commercial suppliers generally don't offer a selection of spontaneously reactive dendrimers for bioconjugation purposes. For this reason, most of the applications published for coupling biomolecules to dendrimers have used various modification or activation steps to create the appropriate reactive groups for conjugation (e.g., Leon *et al*., 1996).

Due to the multivalent nature of dendrimers, the first consideration for conjugating molecules to them is to decide how many modifications should occur on its surface. For some molecules, maximizing the ligand:dendrimer modification ratio may be desirable. An example is in creating sugar-dendrimer derivatives to interact with carbohydrate binding proteins on cell surfaces. Since many sugar-lectin associations are of low affinity, creating a dendrimer conjugate having numerous sugar molecules attached to its surface is advantageous to form multiple interaction points. This approach results in the sugar-dendrimer complex binding to the cell surface with higher avidity than a single sugar derivative would be able to achieve.

However, for other bioconjugation applications, the optimal number of molecules attached to a dendrimer may have to be determined by experimentation. Too many modifications may result in decreased activity of the final conjugate as compared to a similar conjugate made without the use of a dendrimer. For instance, numerous fluorescent molecules can be attached to a G-3 amine-containing dendrimer to provide an enhanced fluorescent conjugate, which is brighter than a single fluorescent molecule for labeling proteins. However, if too many fluorescent molecules are attached, fluorescence quenching may take place and obviate any benefit the use of a multivalent dendrimer may provide toward signal enhancement. Therefore, for any given dendrimer conjugate preparation, some thought must be given to optimizing the number of modifications (or the ligand:dendrimer ratio) to obtain the best possible conjugate activity in the intended application.

In addition, if a dendrimer is to be labeled with one molecule and then ultimately attached to another molecule to form the complete complex, then the second conjugation step also must be planned from the beginning. Are some surface groups going to be used for coupling the first molecule and then the remaining groups used for coupling to the second one or will a disulfide dendritic core be used for coupling to the second molecule after cleavage of the modified dendrimer? Such decisions will affect the conjugation strategies used with dendrimers and often govern the usefulness of the resultant conjugate.

The following methods of linking molecules to dendrimers present options for deciding the best reactions to exploit to create a conjugate. In all cases, the ratio of reactants and the nature of the final conjugate should be carefully considered. In the end, running a series of trial conjugations to optimize the final conjugate will result in a method that is appropriate to the intended application and consistent in performance from batch to batch.

2.1. Coupling to Amine-Dendrimers

With PAMAM (or Priostar) amine-containing dendrimers, the surface is adorned with many primary amine groups, the number of which is dependent on the generational size of the dendritic structure. For instance, a G-3 PAMAM dendrimer has 32 amine groups on the outer ends of its branches, a G-4 has 64 amines, and a G-5 has 128 amines. As long as the dendrimer size is within the mid-range where crowding of surface components is not severe, then the reactivity of these pendent amines is much greater than the amines on a globular protein. This is due to the fact that with protein molecules solvent accessibility of amines is dependent on the relative exposure of the side chain lysine amines to the environment. For many globular proteins, a percentage of amines are highly accessible and react quickly, but for another population of amines, they are somewhat buried below the surface polypeptide structure and don't react as readily (Chapter 1, Section 1.1).

With dendrimers, the pendent amine groups are all approximately equally accessible and very reactive for bioconjugation or modification reactions (Fréchet, 1994). The only potential limitation for coupling to a dendrimer surface would be steric crowding, which would restrict the number of large molecules from coupling to every functional group on the dendrimer. For the coupling of small molecules such as sugars or biotin derivatives, the total number of possible modifications will approach the total number of amine groups on the surface, providing the dendrimer is not so large that there is already severe surface crowding of the branches. For globular protein coupling, however, the number of potential coupling points may be much less than the total number of functional groups available, because each protein molecule will overlap and block some functional groups as it is coupled to the dendrimer surface.

Modification of Amine-Dendrimers with Sulfo-NHS-LC-SPDP

The amines of a PAMAM dendrimer may be reacted with any heterobifunctional crosslinker, which contains an amine-reactive group on one side and another end having different reactivity. The result will form a reactive dendrimer that is useful for coupling proteins and other ligands, which have functional groups able to form a covalent linkage with the second reactive group on the crosslinker. Sulfo-NHS-LC-SPDP contains a sulfo-NHS ester that will couple with the pendent amine groups on the dendrimer and a pyridyl disulfide at its other end to couple with thiol-containing ligands (Chapter 5, Section 1.1). Reaction of this reagent with an amine-containing dendrimer yields a reactive intermediate containing a long aliphatic spacer arm and terminating in reactive pyridyl disulfide groups (Figure 7.9). A disulfide linkage can be formed by reaction of the pyridyl disulfide end with a thiol-containing ligand, which is reversible by reduction with DTT or TCEP.

Another strategy using this crosslinker is purposely to reduce the pyridyl disulfide end after dendrimer modification to create a free thiol on the dendrimer. Singh (1998) used this process to thiolate dendrimers for subsequent coupling with protein molecules containing a group reactive to thiols, such as sulfo-SIAB (or sulfo-SMCC) activated antibodies. After the initial activation, SPDP-PAMAM dendrimers may be stored indefinitely either lyophilized or frozen, because the thiol-reactive group is stable to hydrolysis or degradation. The modified dendrimers also may be treated with DTT to release pyridine-2-thione and create free sulfhydryls on the dendrimer surface. A thiolated dendrimer should be kept in the presence of at least 10 mM EDTA and used

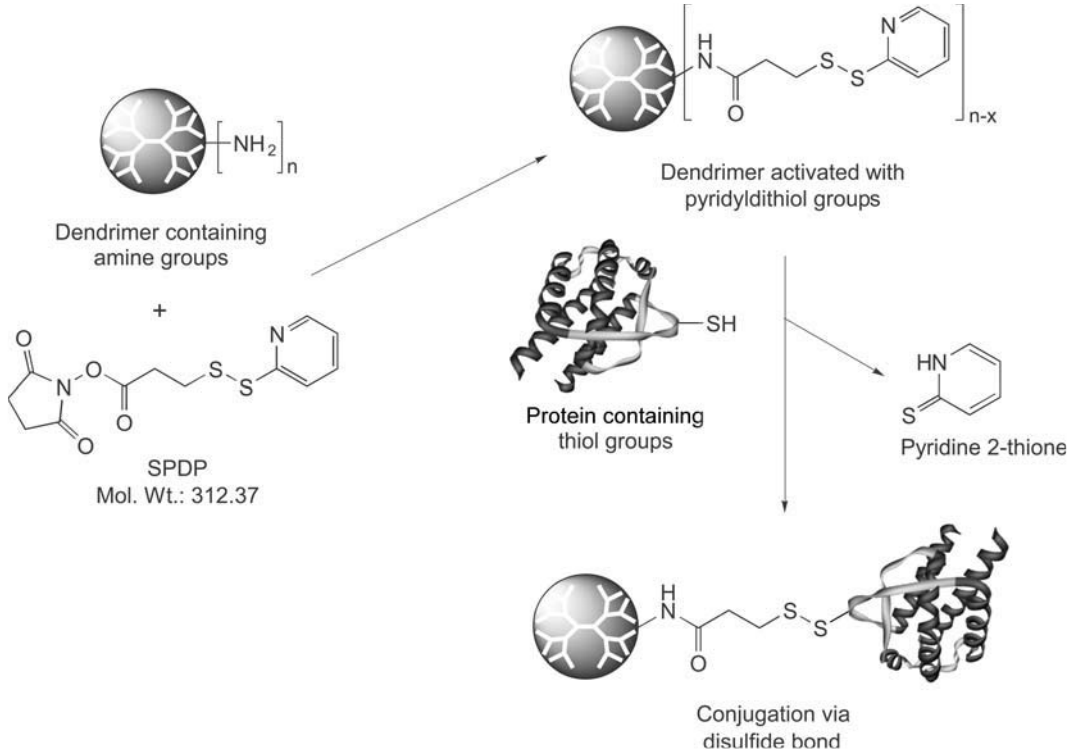


Figure 7.9 Amine-containing dendrimers can be activated with SPDP to create thiol-reactive derivatives. Alternatively, the pyridyl dithiol group may be reduced to create free thiols on the dendrimer surface for subsequent conjugation.

immediately for bioconjugation to prevent oxidation of the thiols, which could form oligomers over time if disulfides are formed between dendrimer molecules.

Use of sulfo-NHS-LC-SPDP or other heterobifunctional crosslinkers to modify PAMAM dendrimers may be done along with the use of a secondary conjugation reaction to couple a detectable label or another protein to the dendrimer surface. Patri *et al.* (2004) used the SPDP activation method along with amine-reactive fluorescent labels (FITC or 6-carboxytetramethylrhodamine succinimidyl ester) to create an antibody conjugate, which also was detectable by fluorescent imaging. Thomas *et al.* (2004) used a similar procedure and the same crosslinker to thiolate dendrimers for conjugation with sulfo-SMCC-activated antibodies. In this case, the dendrimers were labeled with FITC at a level of 5 fluorescent molecules per G-5 PAMAM molecule.

Reaction of a G-3 PAMAM dendrimer with only a 4- to 10-fold molar excess of sulfo-NHS-LC-SPDP was found to yield a partially modified surface containing only a few pyridyl disulfide groups and leaving the rest of the amines available for further conjugation (Singh, 1998). To create 7–10 thiols per dendrimer, the reaction can be carried out using a 20-fold excess of sulfo-NHS-LC-SPDP. The following protocol should be optimized to incorporate the level of thiolation best suitable for the application of the final conjugate.

Protocol

1. Dissolve the amine-containing PAMAM dendrimer in methanol or a buffered aqueous medium at a pH of 7–9 (e.g., 50 mM sodium phosphate, pH 7.5) and at a concentration of at least 10 mg/ml. Note that Singh (1998) used a concentration of 110 mg/ml in methanol, but other dendrimer concentrations should work equally well. For nonaqueous reactions, the addition of a proton acceptor may aid in driving the reaction to maximal yields (i.e., triethylamine or dimethylaminopyridine).
2. Dissolve sulfo-NHS-LC-SPDP at a concentration of 20 mM (5.2 mg/ml) in DMSO or water. If water is used, the solution must be used immediately to prevent hydrolysis of the sulfo-NHS ester.
3. With mixing, add an aliquot of the crosslinker to the dendrimer solution to provide the desired molar excess of reagent. For many applications, less than 10 pyridyl disulfide groups are needed per dendrimer molecule; therefore, molar ratios in the range of 5–20 × excess of crosslinker over the amount of dendrimer present typically are used.
4. React with mixing for at least 30 minutes at room temperature. Longer reactions may be used without problems.
5. Purify the derivatized dendrimer using gel filtration (size exclusion chromatography) on a desalting column or through use of ultrafiltration spin-tubes (for G-4 and above). For smaller dendrimers, the derivatives may be purified by repeated precipitation from a methanolic solution by addition of ethyl acetate, dioxane, or benzene. The SPDP-dendrimer may be dried by lyophilization (if in water or buffer) or by solvent evaporation in vacuo (if the precipitation method was used).

   The SPDP-modified dendrimer of step 5 may be further derivatized with an amine-reactive fluorescent molecule for use in fluorescence detection applications. The fluorophore modification may be done prior to coupling an antibody or other molecules to the dendrimer for targeting purposes. The following steps illustrate the procedure used to obtain the fluorescently labeled dendrimer and then to use the SPDP-modified dendrimer to form thiols on the surface through reduction or to link to another molecule containing a thiol. Step 6 is optional for adding fluorescence detection capabilities, and the protocol of either step 7 or 8 may be used to conjugate the SPDP-reactive group to another protein.
6. Dissolve the purified SPDP-modified dendrimer of step 5 in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.5, or in DMSO at a concentration of at least 10 mg/ml. Add a 10–20 × molar excess of an amine-reactive fluorescent molecule (i.e., NHS-rhodamine or a hydrophilic NHS-Cy5 derivative see section on fluorescent probes). React with mixing for 1 hour at room temperature. Purify the fluorescently labeled SPDP-modified dendrimer using gel filtration or ultrafiltration. Follow the method of either step 7 or 8 to conjugate the dendrimer to another protein or molecule.
7. To create a thiolated dendrimer, the pyridyl disulfide groups may be reduced by addition of 50 mM DTT or TCEP in 50 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5, which will release pyridine-2-thione groups and leave sulfhydryls on the dendrimer surface. Remove excess reducing agent by gel filtration on a column of Sephadex G-25 or the equivalent. The thiolated dendrimer should be used immediately to conjugate to a protein or another molecule containing a thiol-reactive group, such as a maleimide or iodoacetyl group.
8. Alternatively, a thiol-containing protein may be directly conjugated to the SPDP-modified dendrimer to create a disulfide linkage. Add a sulfhydryl-containing protein

or other molecule to the purified SPDP-modified dendrimer in 50 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5. The amount of the thiol-containing protein to be added to the dendrimer should be determined experimentally to be optimal for the intended application of the conjugate. Typically, an excess of thiol-protein over the number of SPDP groups per dendrimer is added to assure that the number of proteins coupled will efficiently utilize the number of modifications initially made using sulfo-NHS-LC-SPDP. Thus, controlling the molar ratio used in the initial crosslinker reaction will control the molar ratio of protein-to-dendrimer in the final conjugate.

NHS-PEG-Maleimide Coupling to Amine-Dendrimers

An alternative method to the use of sulfo-NHS-LC-SPDP for coupling thiol-containing proteins or antibodies to PAMAM dendrimers is to use a heterobifunctional crosslinker containing an amine-reactive NHS ester and a thiol-reactive maleimide group (Chapter 5, Section 1). Unlike the pyridyl disulfide reaction with a sulfhydryl as in the SPDP protocol described previously, a maleimide group forms a stable thioether linkage with a sulfhydryl-containing ligand, which is not cleavable by reduction.

A common choice of crosslinker for this type of reaction is sulfo-SMCC, which has been used extensively for antibody conjugation (Chapter 20, Section 1.1). A better option for dendrimer conjugation is to use a similar crosslinker design, but one that contains a hydrophilic PEG spacer arm to promote dendrimer hydrophilicity after modification. Derivatization of an amine-dendrimer with a NHS-PEG-maleimide can create an intermediate that is coated with water-soluble PEG spacers. This modification helps to mask any potential for nonspecific interactions that the PAMAM surface may have, while providing terminal thiol-reactive maleimides for coupling ligands (Figure 7.10).

If a NHS-PEG-maleimide compound is used for this type of activation and coupling, the intermediate maleimide-activated dendrimer should be quickly purified of excess crosslinker and reaction by-products and immediately used to couple ligand. This is due to the fact that the maleimide hydrolyzes in aqueous solution at a higher rate than an SMCC-type crosslinker, because of the extreme hydrophilicity of the PEG spacer arm compared to the cyclohexane spacer of SMCC.

NHS-PEG-maleimide crosslinkers are available in a number of spacer lengths depending on the size of the polymer chain in the PEG arm. Long chain crosslinkers of this type use PEG polymers of molecular weight approximately 2,000 to 5,000, containing from about 45 to over 100 repeating polyethylene oxide units. Shukla *et al.* (2003) used PEG modifications to increase the half-life of folate receptor-targeted dendrimers for boron neutron capture therapy. However, a major deficiency of such full-length PEG polymers is that they are extremely polydisperse and consist of a broad range of polymer lengths, which makes reproducibility of their size difficult to achieve. Modifying a dendrimer with this type of polymeric crosslinker results in variability of the length of the PEG chains displayed on its surface, which may be detrimental for coupling some ligands. In addition, the use of full-length PEG polymers perhaps should be avoided if the resultant hydrodynamic volume of the dendrimer derivative becomes unacceptably large for some applications.

Alternatively, shorter, discrete crosslinkers containing PEG spacers of known chain length are now available (Thermo Fisher, Quanta BioDesign; see Chapter 18). These reagents are designed to contain an exact number of PEG units, typically from between 2 repeating units

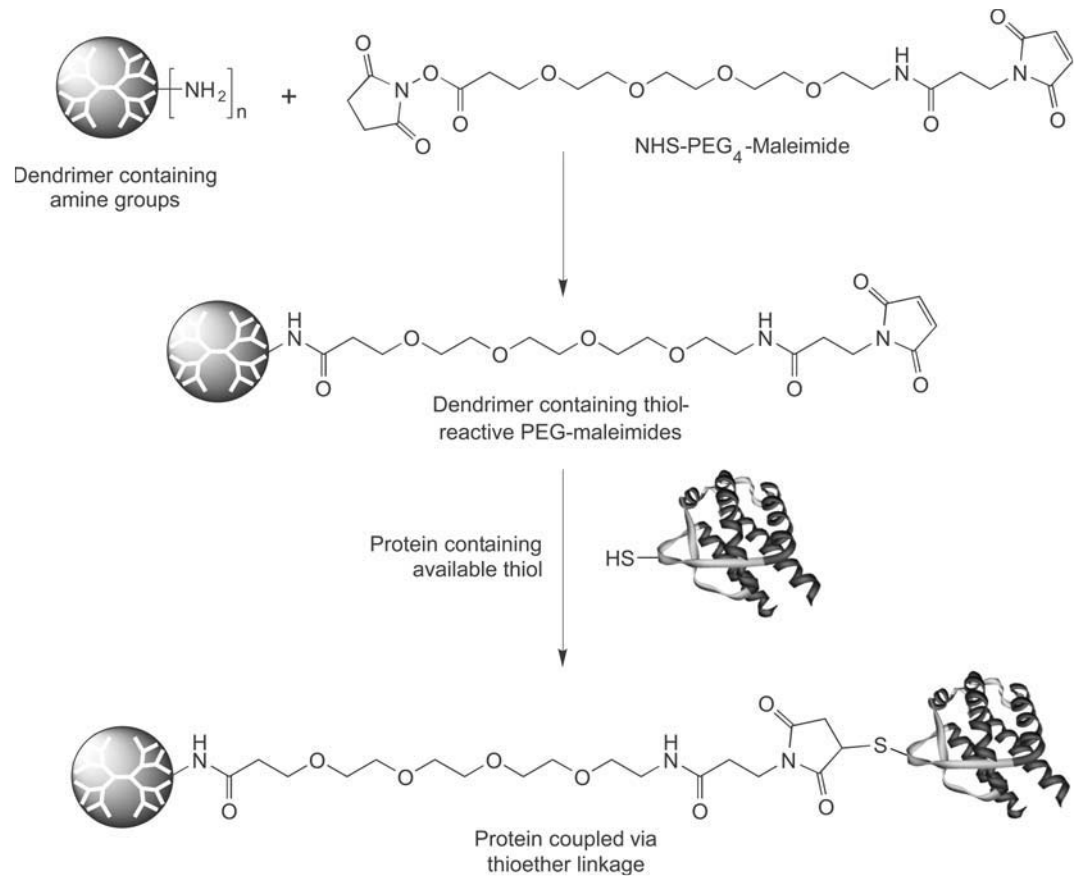


Figure 7.10 An NHS-PEG-maleimide compound can be used to functionalize dendrimers to provide a hydrophilic spacer terminating in thiol-reactive groups. Thiol-containing proteins then can be conjugated to this reactive intermediate to form covalent thioether bonds.

to 24 repeating units. The PEG spacer chain increases the overall water solubility of modified molecules and decreases nonspecific binding potential of the final conjugate. The following protocol may be used with any of these discrete PEG crosslinkers with the appropriate adjustments in the quantity of reagent added to take into account differences in molecular weight due to the PEG length. The longer of these discrete NHS-PEG-maleimide crosslinkers will provide the greatest degree of hydrophilicity after modification of an amine-dendrimer surface. Intermediate-length NHS-PEG-maleimides probably provide a sufficient combination of hydrophilicity while maintaining a smaller conjugate size. One caution should be noted when using PEG-based reagents with amine-containing dendrimers. The polyether cross-bridge of PEG compounds has the ability to hydrogen bond to the dendrimer surface, especially when using dendrimers with a high density of amines. Reactions done at higher levels of PEG-containing reagents may be done to overcome this tendency. Alternatively, the amine surface may be partially blocked or derivatized with another molecule prior to using the PEG compound to avoid hydrogen bond interactions.

Protocol

1. Dissolve 10 mg of an amine-containing dendrimer into 1 ml of 50 mM sodium phosphate, 0.15 M NaCl, pH 7.5 (coupling buffer) with mixing.
2. Dissolve NHS-PEG_6-maleimide (MW 601.6) into DMSO at a concentration of 20 mM. Short, PEG-type crosslinkers often exist as a thick oily mass, and preparing the solution typically involves dissolving an entire vial of the compound into DMSO to determine accurately the required concentration. Use only dry DMSO to avoid hydrolysis of the NHS ester.
3. Add 50 µl of the NHS-PEG_6-maleimide solution to the 1 ml dendrimer solution and mix thoroughly to dissolve. This represents approximately a 14-fold molar excess of crosslinker over the quantity of dendrimer present, if a G-3 PAMAM dendrimer is used with an ethylenediamine core. The optimum molar ratio of crosslinker-to-dendrimer should be determined experimentally for best performance of the resultant conjugate in its intended application. If enough material is available, doing a series of experiments at different mole ratios of crosslinker-to-dendrimer will help to optimize the resultant conjugate.
4. React for 1 hour at room temperature with mixing.
5. Purify the derivatized dendrimer from excess crosslinker and reaction by-products using gel filtration (size exclusion chromatography) on a 10 ml desalting column or through use of ultrafiltration spin-tubes. If a dendrimer of at least G-3 size is being modified, the separation should be done on a support with an exclusion limit of no more than 2,500–5,000 Daltons to avoid losing the derivatized molecule through the membrane or not obtaining sufficient separation during the chromatography.
6. Add 1–10 mg of a protein or antibody containing an available thiol group to the purified, modified dendrimer from step 5. Alternatively, add the protein to be coupled to the dendrimer suspension in an amount equal to 1–10 × molar excess over the estimated number of maleimide groups on the modified dendrimer. The amount of maleimide functionality may be determined using the protocol in Chapter 19, Section 5. Creating thiol groups from disulfides in proteins may be done according to the procedures in Chapter 1, Section 4.1. Alternatively, the use of a thiolation reagent may be done to add thiols to the protein surface for coupling to the maleimide groups. The optimal amount of protein to be added to the dendrimer should be determined experimentally.
7. React the protein with activated dendrimer for 2 hours at room temperature with mixing. At the completion of the reaction, cysteine may be added at 50 mM to block excess maleimide-reactive sites, which are not coupled with protein.
8. Purify the conjugate and remove excess protein by gel filtration using a column with an exclusion limit that is able to accommodate both the protein being conjugated and the dendrimer-protein conjugate. If the conjugate elutes in the void volume, while the protein is retained in the pores, this also will result in sufficient separation to purify the conjugate. Store the dendrimer conjugate frozen (especially if it's a PAMAM-type) or lyophilized. The addition of a stabilizing excipient to the freeze-dried conjugate may be done to protect the coupled protein during lyophilization (i.e., sucrose).

Coupling Glycoproteins to Amine-Dendrimers by Reductive Amination

The amines on the surface of PAMAM-type and other amine-containing dendrimers may be used to couple to aldehyde groups in other molecules, including those formed after periodate

oxidation of sugar groups in glycosylated proteins. Mild treatment of glycoproteins with sodium meta periodate results in cleavage of diol carbon–carbon bonds with concomitant oxidation of the hydroxyls to aldehyde groups. These aldehydes can be used to conjugate the proteins to amine-dendrimers through Schiff base formation and reduction to secondary amine linkages using sodium cyanoborohydride (Chapter 1, Section 4.4 and Chapter 2, Section 5) (Figure 7.11).

The following protocol involves the conjugation to an amine-dendrimer of a periodate-xidized glycoprotein, such as an antibody, which has been treated to produce aldehydes according to the protocols in Chapter 1, Section 4.4. This type of conjugation reaction

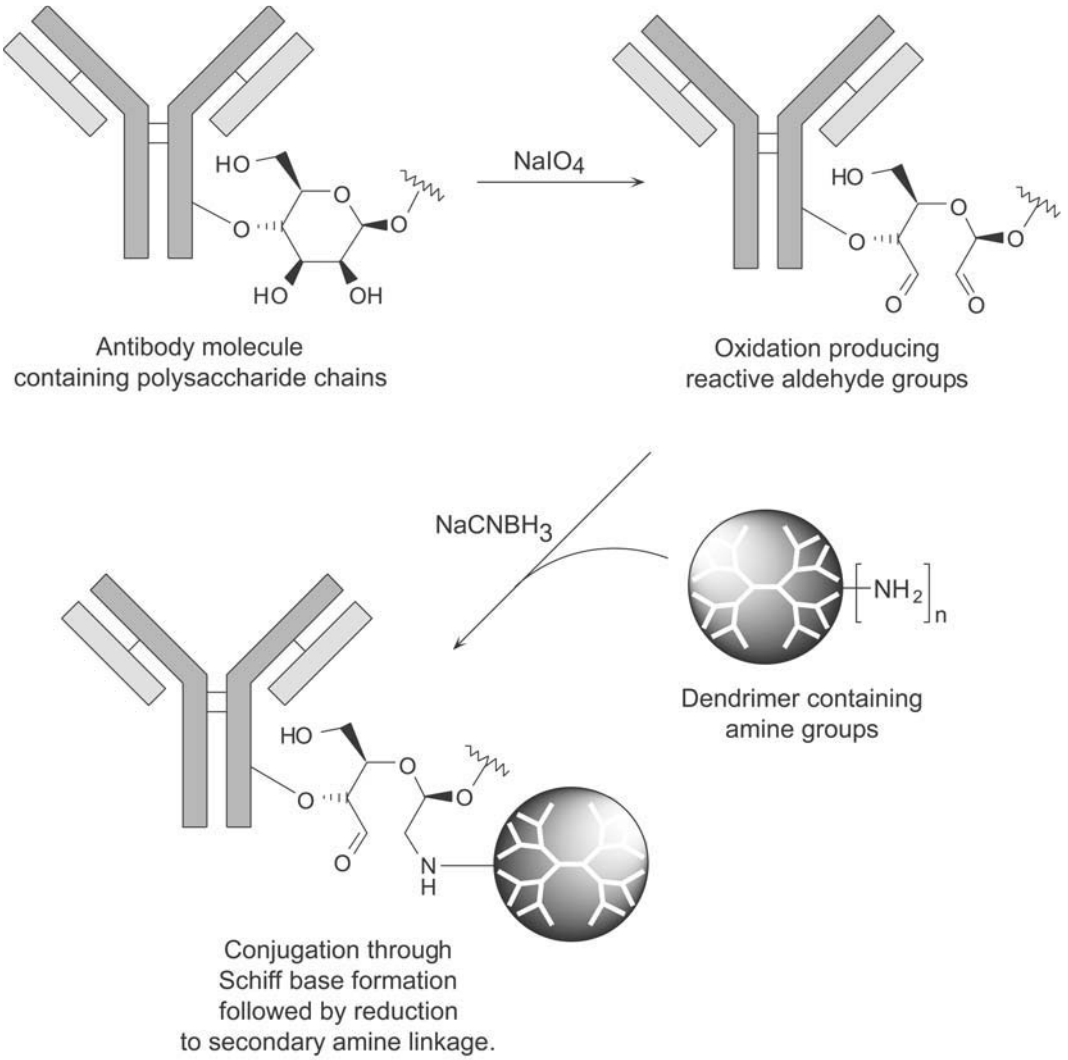


Figure 7.11 Oxidation of glycoproteins with periodate, such as glycosylated antibodies, results in the formation of aldehyde groups that can be used for conjugation to dendrimers containing amine groups. Reductive amination with sodium cyanoborohydride results in coupling via secondary (or tertiary) amine bonds.

to dendrimers may be used even after the dendrimer has been initially modified with a limited number of heterobifunctional crosslinking molecules. Thus, an amine-dendrimer first may be reacted with crosslinkers such as sulfo-NHS-LC-SPDP or NHS-PEG_6-maleimide as described previously, and the remaining amines on the dendrimer surface used to couple with an oxidized glycoprotein. This would create that contained a covalently linked glycoprotein with thiol-reactive groups available for further conjugation with another molecule containing a sulfhydryl group. Singh (1998) used this technique to produce a dendrimer conjugate containing alkaline phosphatase for detection and a Fab′ fragment of an antibody directed against creatine kinase MB isoenzyme. The phosphatase enzyme was coupled through its carbohydrates by reductive amination and the Fab′ fragment was coupled through a thiol group.

Protocol

1. Dissolve an amine-containing dendrimer in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of at least 10 mg/ml. *Note*: The use of a buffer at pH 9–10 (i.e., 0.1 M sodium carbonate) for the initial Schiff base formation (step 2) will result in higher efficiency of conjugation. However, if a higher pH is used during this first stage, then the pH must be adjusted back down to more neutral conditions before the addition of reductant (step 4), as the reducing agent is not effective in the higher pH environment.
2. Add a quantity of a periodate-oxidized glycoprotein to the dendrimer solution (made according to Chapter 1, Section 4.4) to provide a molar ratio of protein-to-dendrimer of 1:1–10:1. Mix to dissolve. The optimal level of protein addition should be adjusted to provide maximal performance and activity in the intended application. If another protein also is to be attached to the dendrimer to make the final complex, then limiting the density of the periodate-oxidized protein may be necessary to leave room on the surface for additional protein coupling. In addition, some proteins with multiple glycosylation sites may cause dendrimer oligomerization if the conjugation reaction is done with too low of a protein-to-dendrimer ratio.
3. React for 2 hours at room temperature with mixing.
4. In a fume hood, add 10 µl of 5 M sodium cyanoborohydride (Sigma) per ml of reaction solution. Caution: cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 M stock solution is dissolved in 1 N NaOH. If a higher pH buffer was used for the Schiff base formation, then adjust the solution to pH 7.5 before adding the cyanoborohydride.
5. React for 30 minutes at room temperature in a fume hood.
6. Block unreacted aldehydes by the addition of 50 µl of 1 M ethanolamine, pH 7.5, per ml of reaction solution. Block for 30 minutes at room temperature with mixing.
7. Purify the conjugate from unreacted protein or unreacted dendrimer using gel filtration chromatography with a matrix having an exclusion limit appropriate to accommodate the size of the molecules being separated (i.e., a HiPrep 16/60 column packed with Sephacryl S-200 HR, GE Healthcare).

Blocking of Amines on PAMAM Dendrimers

The number of amine groups on dendrimers of medium to large size (G-3 and above) often is many more than what is required to prepare a conjugate. Since amines can become protonated

and carry a positive charge even under physiological conditions, they may interact nonspecifically with biomolecules. Ionic interactions can be a significant source of high backgrounds in assays or create off-target effects if using a dendrimer conjugate in the development of *in vivo* applications (cell-based or whole animal). In particular, nonspecific binding to the positively charged amine groups on dendrimers, if left unblocked, can be cytotoxic to normal cells *in vivo* especially if the conjugate is designed to carry chemotherapeutic components, which can be used to kill cancer or diseased cells (Patri *et al*., 2002; Kukowska-Latallo *et al*., 2005). To overcome the positive charge character of amine-dendrimers, excess amine groups can be covalently blocked to eliminate the possibility of ionic interactions. This blocking process can occur before activation of the dendrimer with a crosslinker or after modification, providing that the blocking agent used to couple with the remaining amines does not affect the activation chemistry.

Singh (1998) used succinylation with succinic anhydride to block excess amines and convert them into negatively charged carboxylates, which often have lower interaction potential with biomolecules than positive charges. Thomas *et al*. (2004) used acetic anhydride to acetylate and block the amines of a G-4 PAMAM dendrimer before or after activation with sulfo-NHS-LC-SPDP. Similarly, Patri *et al*. (2004) also used acetic anhydride to block amines on a G-5 PAMAM dendrimer prior to activation with sulfo-NHS-LC-SPDP, but the blocking step was done to eliminate about 80 of the 128 amine groups before effecting the conjugation with an antibody. In all these instances, the amine groups on the dendrimer are converted into amides, which carry no charge at physiological pH.

Reactions with succinic anhydride or acetic anhydride to block dendrimer amines can be done in aqueous or methanolic solution. If organic solvent is used for the reaction, then it is typical to include triethylamine as a proton acceptor, which helps drive the reaction. Such reactions, however, can't be done to dendrimer amines once a protein containing amines also has been conjugated, as the protein too will get modified.

Another method of blocking excess amines on dendrimers is to use small, hydrophilic blockers that contain hydroxyl or ether groups. Islam *et al*. (2005) used the short epoxy compound glycidol to modify amine groups to reduce nonspecific interactions in a dendrimer conjugate. The primary amine groups can be alkylated a maximum of 2 times with this reagent in methanol, resulting in 4 hydroxyl groups for each amino functionality (Shi *et al*., 2007). The reaction with glycidol, however, was shown to be not as efficient in creating a homogeneous product as acylation with either succinic anhydride or acetic anhydride (Shi *et al*., 2005). This likely is due to steric issues coming into play in trying to alkylate each amine twice with glycidol as opposed to a single acylation product with either anhydride compound. Glycidol modification of an amine-dendrimer is done according to the following protocol.

Protocol

1. Dissolve the amine-dendrimer to be modified in methanol at a concentration of 10 mg/ml (all operations are done in a fume hood).
2. Add glycidol to the dendrimer solution in a 4-fold molar excess over the number of amines to be blocked (Shi *et al*., 2007). The number of amines may be calculated from the generation number of the dendrimer and the degree of blocking desired. Obtaining the optimal molar ratio for a particular conjugate application may have to be done by experimentation using a series of different glycidol-to-dendrimer molar ratios.
3. The reaction is continued for 24 hours in the fume hood with mixing.

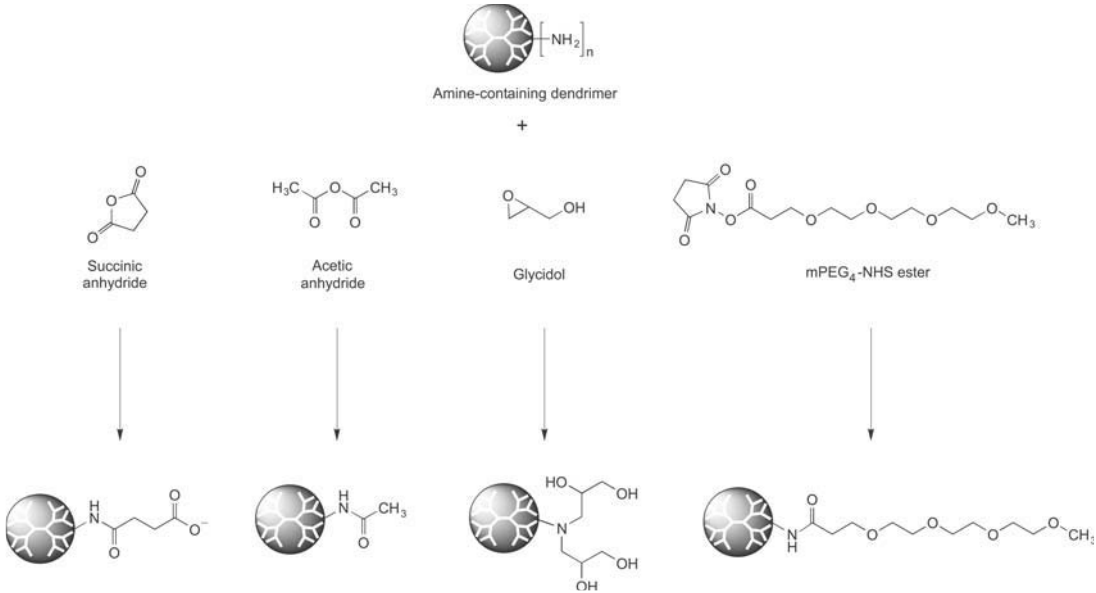


Figure 7.12 Amine-containing dendrimers can be modified using a number of common reactive modification agents. Excess amine groups can be blocked using acetic anhydride, glycidol, or an NHS-mPEG compound. Amines also can be converted into carboxylates using succinic anhydride.

4. Dialyze the modified dendrimer against PBS buffer and then water to remove excess reactants.

Another option to limit the nonspecific binding character of amine-dendrimers is the use of PEG compounds. Amine-reactive PEG derivatives can be used to covalently link to excess amines on dendrimers and create a hydrophilic tether, which can dramatically limit nonspecific interactions with the exposed surface. For this purpose, PEG reagents containing an activated carboxylate on one end and a methyl ether group (mPEG) on the other end work well (Chapter 18, Section 4). NHS-mPEG derivatives that are spontaneously reactive to the dendrimer surface amines will form amide bonds and eliminate the positive charge character of the pendent amino groups. Such PEG compounds may be a superior option to either succinylation or acetylation of amines to eliminate charge, because the PEG component also adds considerable hydrophilicity and low interaction potential to the resultant dendrimer derivative (Figure 7.12).

Acylation reactions to block amine groups on PAMAM dendrimers with anhydride compounds are done in a similar manner to glycidol modification. The following protocol is based on the method of Majoros *et al.*, 2005.

Protocol

1. Dissolve an amine-containing dendrimer in methanol at a concentration of 15 mg/ml with mixing. All operations should be done in a well-ventilated fume hood. A G-5 dendrimer was used in this reaction by Majoros *et al.* (2005), which contained 110 available amine groups.

2. Add to the dendrimer solution a quantity of acetic anhydride that represents a molar ratio of anhydride-to-amines of 0.72:1.0 (680 µl of acetic anhydride was added for the G-5 dendrimer). Using a molar quantity of anhydride that is less than the amount of amines present on the dendrimer assures that only a portion of the amines will become blocked, so that further modification remains possible.
3. Add a quantity of triethylamine to the solution so that a 25 percent molar excess over the amount of anhydride will result (1.25 ml for the G-5 dendrimer).
4. React for 2 hours at room temperature with mixing (in a fume hood).
5. Extensively dialyze the reaction mixture against water or buffer to remove excess reactants.

Preparation of Sugar-Dendrimer Derivatives

The multifunctional nature of an amine-dendrimer can be used to advantage to mimic multidentate interactions of molecules with cell surfaces or virus particles. For instance, the binding affinity of carbohydrate binding proteins (lectins) for individual sugar molecules typically is weak, on the order of 10^6 M^{-1}. In the native method used to increase the binding strength of these interactions, lectins on cell surfaces usually engage in multipoint attachments with carbohydrates or glycans. The conjugation of sugars to the pendent amine groups on dendrimers provides a scaffold for similar multi-site interactions with lectins, which effectively increases the avidity of the resultant binding complex (Aoi *et al*., 1995; Bertozzi and Kiessling, 2001). This design is structurally similar to the multiple tree branched structures of glycans on glycoproteins. The terminal sugar groups on such glycoconjugates are capable of interacting with more than one binding site or more than one receptor on cell surfaces. This effectively turns a single low affinity interaction into a high affinity binding event, which forms the basis for many life processes, including cellular recognition, adhesion, transport, and cell signaling (Clarke and Wilson, 1988; Sharon and Lis, 1989). For a review of glycobiology, see the entire issue of Science: Carbohydrates and Glycobiology, Vol. 291, March 23, 2001, pages 2263–2502.

A series of different mannose-dendrimers was synthesized to investigate their interaction with Concanavalin A (Con A) (Woller and Cloninger, 2002; Woller *et al*., 2003). It was discovered that the sugars on the dendrimer surface were able to bind to the Con A binding sites just like free methyl mannose in solution. However, as the size of the dendrimer increased and number of multivalent mannose residues became available for binding to multiple Con A interaction sites, the affinity of the interaction dramatically increased. For a G-3 mannose-dendrimer derivative, the sugar complex was about 45 times more active than methyl mannose in solution. For larger sized mannose-dendrimer complexes, the increase in activity of binding was up to 660-fold greater than free methyl mannose. However, the interaction potential for the mannose-dendrimer derivatives also was shown to be dependent on the degree of mannose loading. For large dendrimers, steric crowding of sugar molecules on the dendrimer surface decreased its binding activity toward Con A if the mannose loading was greater than about 50 percent of the amines modified. Thus, both dendrimer size and the level of modification must be carefully considered when designing sugar-dendrimer conjugates.

Such sugar-dendrimer complexes ("sugar balls") have been used to inhibit the interactions of viruses with cell surfaces. Many viruses bind to particular carbohydrate residues on cell surfaces, which in turn facilitate their entry into cells and the resultant infection process. A virus particle presents a multi-dentate surface consisting of many carbohydrate-binding proteins able to interact with multiple cell-surface carbohydrates. The surface of a dendrimer that is modified with

sugar molecules is able to mimic the glycan rich field on the surface of a cell. In this way, sugar-dendrimer complexes are able to interfere with virus binding to cellular targets. For instance, Borges *et al.* (2005) used dendrimers modified with a carbohydrate usually found on immune cell-surface glycosphingolipids to function as an inhibitor of HIV-1 infection. The polyvalent nature of the sugar-dendrimer derivative was able to bind effectively to the virus particles with high avidity, thus preventing the virus from binding to the carbohydrates on the immune cell surface (Borges and Schengrund, 2005). The sugar-dendrimer complex in the vaginal ointment Vivagel, developed by Starpharma, is undergoing clinical trials as a preventative for HIV infection (Halford, 2005). The dendrimer formulation also is said to be active against other STDs, including chlamydia, herpes simplex virus, hepatitis B, and human papilloma virus.

Sugar-dendrimer complexes also may be used as affinity ligands for purification of carbohydrate or sugar-binding proteins. Szwergold *et al.* (2001) used a polyvalent fructose-dendrimer derivative immobilized on a chromatography matrix to purify human fructosamine-3-kinase. The ligand was coupled to the support through excess amine groups on the dendrimers, which were not coupled with sugar molecules, and gel functioned with high affinity toward binding the enzyme out of complex samples.

The synthesis of sugar-dendrimer derivatives may be done using a number of reaction strategies. Aoi *et al.* (1995) used the lactone derivatives of lactose and maltose to react with the terminal amine groups on G2–G4 PAMAM dendrimers. The reaction of a lactone with an amine involves nucleophilic attack on the lactone carbonyl with ring opening to form an amide bond (Figure 7.13). The conjugation was done in organic solvent (DMSO), but the reaction also may be done in aqueous buffers under alkaline conditions.

The preparation of sugar-dendrimer conjugates may encompass a variety of modification levels and sizes, depending on the generation of dendrimer used and the density of sugar molecules modifying its surface. Interactions with lectins vary according to the optimal sugar-to-sugar distance within the final complex. For instance, it is known that the 3 binding sites on the mammalian hepatic lectin for interaction with galactose and *N*-acetylgalactosamine are arranged on the protein in a triangle with a separation of 1.5 nm, 2.2 nm, and 2.5 nm (Lee *et al.*, 1984). Aoi *et al.* (1995) found that the molecular distances between sugar molecules on a G-3 PAMAM dendrimer prepared according to their protocol was between 1.3 nm and 2.9 nm. Thus, the sugar ball dendrimer had an optimal inter-sugar spacing to interact with the binding sites on the targeted lectin.

The following protocol represents the method of Aoi *et al.* (1995) for the coupling of lactone sugars to amine-dendrimers in organic solvent.

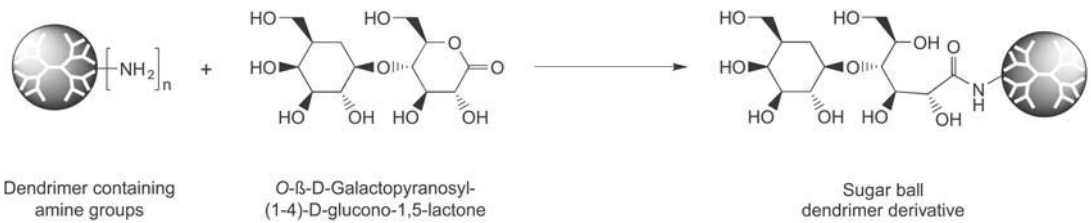


Figure 7.13 Lactone sugar derivatives can be used to react with amine-containing dendrimers, which results in coupling via amide bonds. The "sugar ball" dendrimers then can be used to specifically bind to carbohydrate binding proteins.

Protocol

1. In a fume hood, dissolve 270 mg of a G-4 dendrimer (PAMAM or other amine containing) in 2 ml of dry DMSO. Maintain a nitrogen blanket over the reaction to prevent oxidation. The use of a 3-necked flask and a heating mantle is recommended to control mixing and the proper temperature.
2. Add with mixing, a 300-fold molar excess of a sugar lactone derivative, such as *o*-b-D-galactopyranosyl-(1 → 4)-D-glucono-1,5-lactone (2.6 gm) dissolved in 3 ml of DMSO.
3. React at 40°C for 9 hours or overnight with stirring.
4. After cooling to room temperature, the sugar-dendrimer derivative may be precipitated with a large volume of methanol. The precipitate may be purified from reaction products by dialysis against water or buffer using a membrane with a molecular weight cutoff of 3500 Daltons. The final product may be stored frozen or lyophilized to a white powder.

The modification of amine-dendrimers with lactose also can be done using mono (lactosylamido) mono(succinimidyl)suberate (Thermo Fisher). The amine-reactive compound contains a lactose group at the end of a suberate bridge and terminates at the other end in an NHS ester (Vetter *et al*., 1995). The NHS ester spontaneously reacts with the amine groups on a dendrimer at neutral or slightly basic pH values to form an amide bond (Figure 7.14). The presence of the lactose disaccharide provides a hydrophilic modifying group that maintains the water solubility of the resultant conjugate.

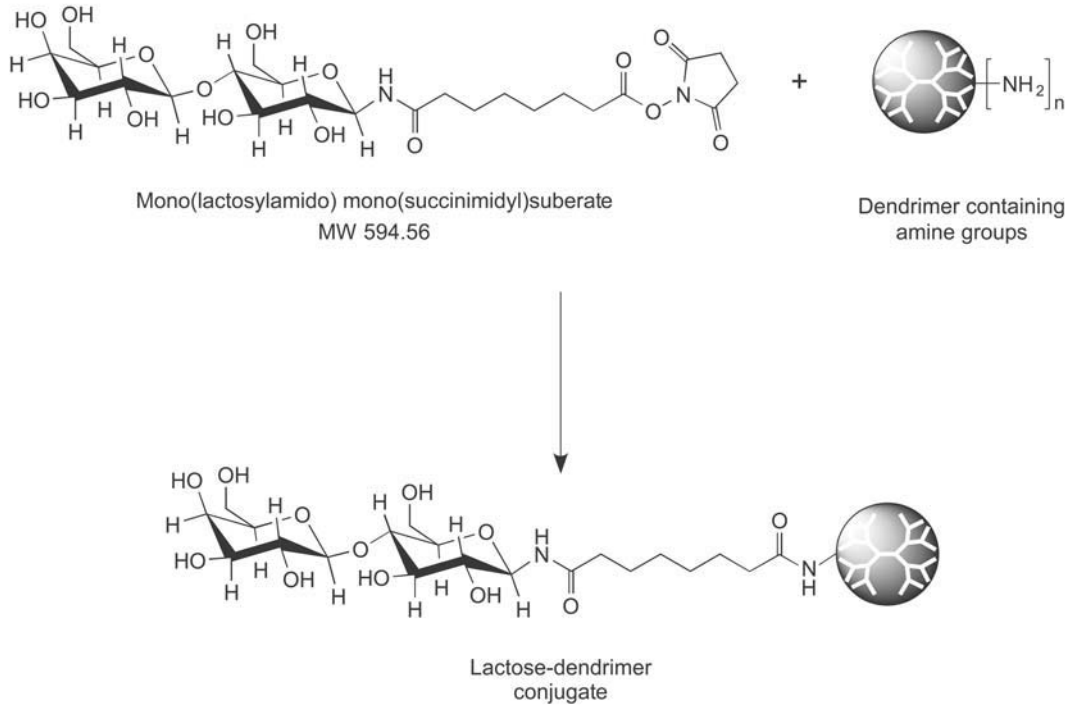


Figure 7.14 The reaction of an amine-containing dendrimer with mono(lactosylamido) mono(succinimidyl)suberate results in the attachment of a lactose unit via an amide bond.

Protocol

1. Dissolve mono(lactosylamido) mono(succinimidyl)suberate in dry DMF to prepare a concentrated stock solution. The compound is extremely soluble in DMF, and solutions of 100 mg/ml may be prepared.
2. Prepare the amine-dendrimer to be glycosylated in a buffer at a slightly basic pH (avoid amine-containing buffers, such as Tris or imidazole). The use of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 works well for NHS ester reactions. The concentration of the dendrimer in the reaction buffer should be at least 10 mg/ml. Other dendrimer concentrations also will work, but highly dilute solutions will result in less efficient modification yields.
3. With mixing, add a quantity of the mono(lactosylamido) mono(succinimidyl)suberate in dry DMF to the dendrimer solution to result in at least a 10- to 20-fold molar excess of reagent over the quantity of amines to be modified in the dendrimer. Depending on the desired application for the lactosyl-modified dendrimer, several different molar ratios may have to be tried to optimize the resulting modification level.
4. React for 30–60 minutes at room temperature with gentle mixing.
5. Purify the modified dendrimer away from reactants and reaction by-products using dialysis or size exclusion chromatography.

Another method for coupling carbohydrates or sugar derivatives to amine-dendrimers is to take advantage of the reducing end of sugars to reductively aminate the amines on the dendrimer surface. This reaction will form stable secondary amine linkages between carbon-1 of the sugars and the dendrimer (Figure 7.15). Saccharides with reducing ends have carbonyl groups that can be coupled to an amine-dendrimer in the presence of a reducing agent. However, since most reducing ends of sugars or glycans exist mainly in an acetal (or ketal) ring form, and only the open form with the exposed aldehyde can participate in a reductive amination reaction, the rate of modification by this process can be slow. The open form of a reducing sugar is available at any given time in only a small percentage of the total saccharide present, usually far less than 1 percent. For this reason, reactions done with primary amines at room temperature may take days to reach acceptable coupling yields. Typically, such reactions are done at elevated temperatures and over the course of at least several days, or sometimes weeks, to reach completion.

The following method for carbohydrate conjugation to dendrimers may be used to couple a variety of reducing sugars to amine-dendrimers, including saccharides, longer-chain carbohydrates, and even complex glycans after release from a protein (see Chapter 1, Section 4.6).

Protocol

1. Dissolve a carbohydrate, saccharide, or glycan sample having a free reducing end in 0.1 M sodium acetate, pH 5.0. Alternative coupling conditions that can be used for the modification reaction include 30 percent glacial acetic acid in DMSO (v/v) or acetic acid/pyridine (1:2, v/v), if the dendrimer is soluble in these solutions. The use of DMSO or pyridine often facilitates solubilization of a greater range of carbohydrates or glycans than aqueous buffers. The presence of acetic acid has been found to accelerate the reductive amination reaction when the organic solvent conditions are used (Bigge *et al.*, 1995). A well-ventilated fume hood should be used for all organic reactions. The concentration of the carbohydrate should be 5–100 μM for glycans, but for the modification of dendrimers with other more abundant carbohydrates, higher concentrations should be

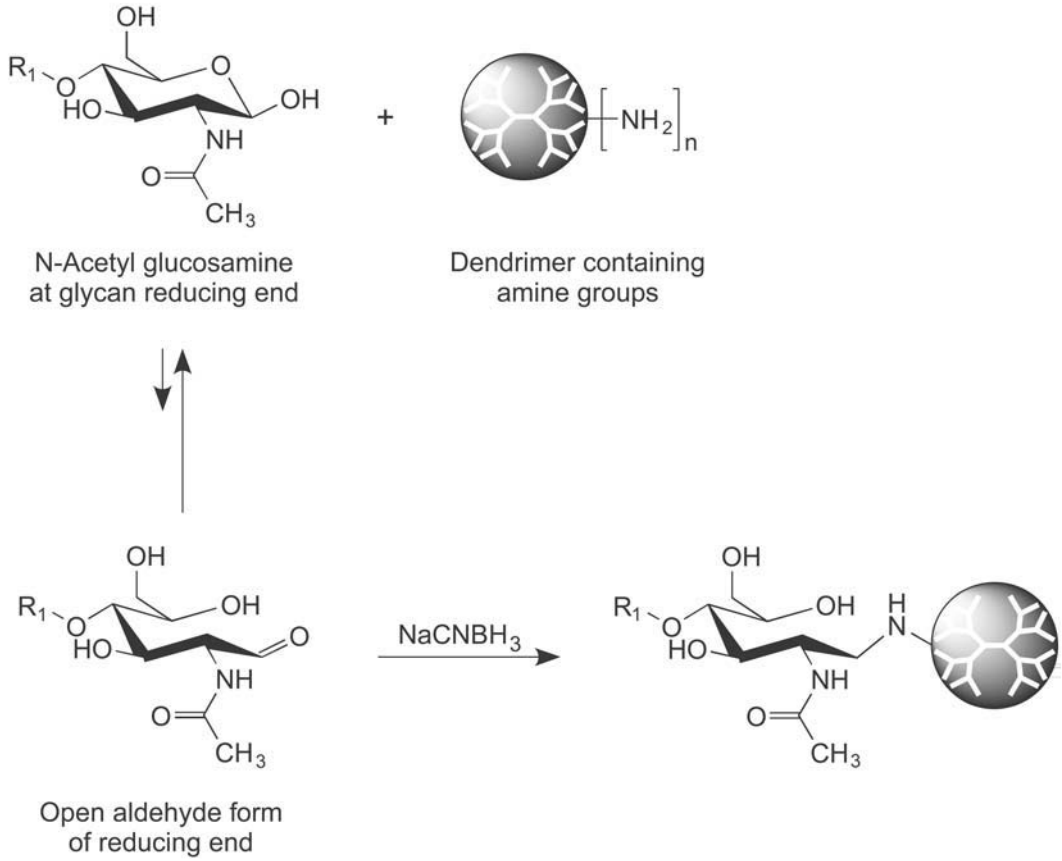


Figure 7.15 The reducing end of a glycan or a carbohydrate can be used to conjugate to an amine-dendrimer by reductive amination, which results in the formation of a secondary amine linkage.

used. For modification of amine-dendrimers that are not soluble in the recommended organic solvents, the glycan or carbohydrate initially may be solubilized in DMSO and then an aliquot added to the aqueous reaction buffer.

2. Add to the glycan solution the amine-dendrimer to be labeled. Dendrimer concentrations of at least 10 mg/ml will help to increase the reaction kinetics, although the reaction proceeds slowly, mainly due to the limited quantity of open-form reducing sugar available for coupling at any given time.
3. In a fume hood, add to the reaction mixture a quantity of reducing agent (e.g., sodium cyanoborohydride or borane dimethylamine (BDA)) to give a final concentration of 1.0 M. The high concentration of reductant will aid in accelerating the reaction to completion. Cyanoborohydride is extremely toxic and should not be handled outside of a fume hood. Use proper protective clothing when handling such compounds.
4. When using nonaqueous reaction conditions, incubate for 1–2 hours at 60–80°C. For reactions in an aqueous environment, the reaction may be done at room temperature or 37°C. In this case, the reaction time should be extended to at least 24 hours. Longer

reaction times are not unusual when modifying carbohydrates by reductive amination at ambient temperature.

5. Purify the modified dendrimer from reactants and reaction by-products by dialysis, gel filtration, or ion-exchange chromatography.

Synthesis of other reactive groups on sugars for conjugation with amine-dendrimers also has been done with success. For example, André *et al.* (1999) used the *p*-isothiocyanato derivative of *p*-aminophenyl-β-D-lactoside to couple with the amine groups on a G-3 PAMAM dendrimer, forming isothiourea linkages between the sugar and dendrimer. Davis *et al.* (2005) used glycosyl derivatives containing the thiol-reactive group methanethiosulfonate to couple with dendrons modified with thioacetic acid to contain terminal thiol groups. This reactive functionality was again used to conjugate to thiol-containing proteins to create glyco-dendrimer-protein reagents. Woller and Cloninger (2001) used another isothiocyanate derivative of mannose to couple with amine-dendrimers of several different generations. Finally, Kensinger *et al.* (2004a) synthesized a thiopropionic acid derivative of galactose and coupled it to amine-dendrimers using the amide bond forming agent HATU (Aldrich) in acetonitrile. The galactose-functionalized dendrimers were used to study their binding to HIV-1 gp120 protein (Kensinger *et al.*, 2004b).

Conjugation of Carboxylate Organic Molecules to Amine-Dendrimers

Amine-dendrimer molecules of various generations have been used as carriers for small organic molecules, such as chemotherapeutic agents or small ligand, cell-targeting compounds, such as folic acid (Quintana *et al.*, 2002; Shukla *et al.*, 2003; Islam *et al.*, 2005). The presence of folic acid on the dendrimer surface allows the drug complex to bind with over-expressed folate receptors on certain tumor cells (Ross *et al.*, 1994). Adding a chemotherapeutic drug to the folate–dendrimer conjugate creates a toxic payload deliverable directly to the cancer cells *in vivo*.

Majoros *et al.* (2005) synthesized such dendrimer conjugates using the carbodiimide EDC to activate the carboxylate group of folic acid or methotrexate for reaction with the amines on the PAMAM dendrimer surface. A G-5 amine-dendrimer first was partially blocked by acetylation according to the previously described procedure. The result was about 82 of the available amines were capped, leaving about 28 amines still free for coupling with other molecules. In some derivatives, a fluorescent probe (FITC) also was conjugated to the partially blocked dendrimers to provide a detectable tag for following binding to cells. Then both folic acid and methotrexate were conjugated to the labeled dendrimer in separate reactions to yield the final conjugate. Careful tuning of the molar ratios used during each reaction resulted in a modification level per dendrimer of 4 FITC labels, 4 folic acids, and 5 methotrexate groups. In some cases, glycidol also was used to block about 14 of the amine groups and form hydrophilic hydroxylated regions on the dendrimer surface to promote water solubility. Figure 7.16 illustrates the final conjugate composition with a G-5 dendrimer. These conjugates were extensively studied by HPLC to determine properties and modification levels (Islam *et al.*, 2005).

Hong *et al.* (2007) investigated the binding affinity of such folate–dendrimer derivatives toward the folate-binding protein (FBP), typically over expressed on cancer cells. Using SPR analysis, they were able to measure the binding constants of a series of folate–dendrimer conjugates containing different levels of folate modification (from 2 to 14 folates–dendrimer). The results indicate that the combined avidity of multiple folates on a single dendrimer enhanced the binding to FBP by up to 5 orders of magnitude (2,500- to 170,000-fold enhancement). This is

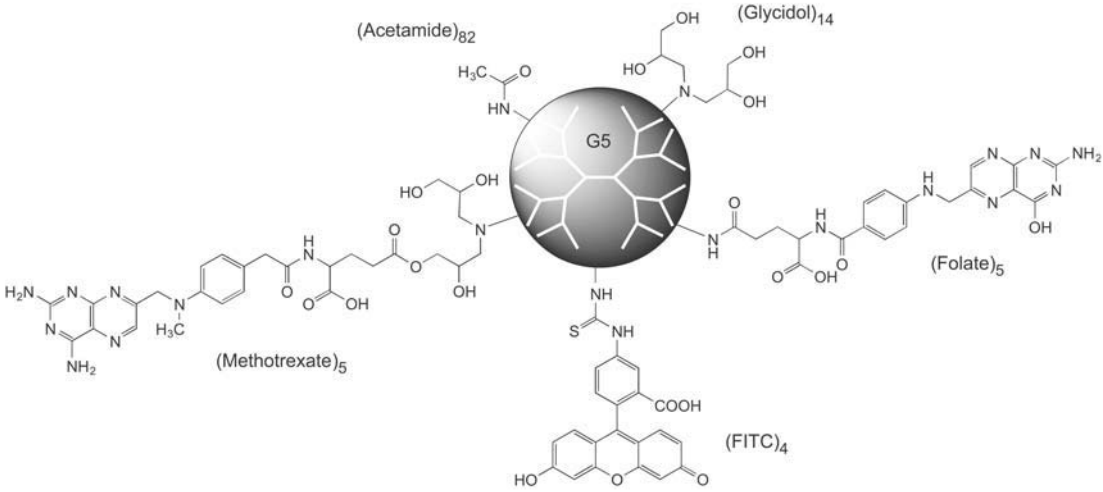


Figure 7.16 The creation of a tumor-targeting dendrimer conjugate can take advantage of the multivalent character of the dendrimer polymer. This figure illustrates the attachment of five different groups to an amine-dendrimer to produce a chemotherapeutic construct.

the first definitive study on the benefits of multivalent dendrimer drugs as compared to the same drug interactions individually in solution. Using this approach to drug targeting, Kukowska-Latallo *et al.* (2005) found that a dendrimer conjugate improved the therapeutic response toward tumor cells in animal models of human epithelial cancer.

Similar dendrimer conjugates for therapeutic application also have been designed for boron neutron capture therapy in the treatment of cancer (Barth *et al.*, 1994; Newkome *et al.*, 1994; Qualmann *et al.*, 1996). In one design, a G-3 PAMAM dendrimer was modified with folate and 12–15 polyhedral decaborate clusters (Alam *et al.*, 1989) along with an average of 1 to 1.5 PEG_{2000} units to make the therapeutic conjugate (Shukla *et al.*, 2003). The folate-targeting component was not attached to the surface amines, but to the end of an additional amino-PEG_{800} unit. The PEG groups also facilitated increased *in vivo* half-life and prevented immediate uptake of the complex into the liver.

Most of these conjugation reactions involve amide bond formation between a small, organic molecule containing a carboxylate and the amines on the dendrimer surface. There are two potential reaction strategies for creating such conjugates: aqueous or nonaqueous reactions. For the coupling of folic acid to PAMAM dendrimers, Majoros *et al.* (2005) used an organic solvent environment according to the following protocol.

Protocol

1. Dissolve 33 mg of folic acid in a mixture of 24 ml DMF and 8 ml DMSO at room temperature.
2. Add a 14-fold molar excess of EDC (200 mg) with mixing.
3. React for 1 hour at room temperature. The product of this reaction forms an amine-reactive ester on folate for coupling to the dendrimer.

4. Dissolve an amine-containing dendrimer in water at a concentration of at least 4.5 mg/ml. Majoros *et al.* (2005) used 403 mg of a partially acetylated G-5 dendrimer that contained 82 blocked amines and 28 available amines for coupling, dissolved in 90 ml of water.
5. Slowly add with mixing, the activated folic acid from step 3 to the amine-dendrimer solution to give a final molar ratio of folate-to-dendrimer of 5.5:1.
6. React for 1 hour at room temperature with mixing.
7. Purify the modified dendrimer from excess reactants and reaction byproducts by dialysis against water or buffer.

The same type of modification with carboxylate molecules can be done in aqueous solution using EDC. If the ligand to be coupled only has a single carboxylate with no amines or other nucleophiles present, then the dendrimer and ligand may be dissolved at a similar molar ratio in aqueous buffer and EDC added to facilitate the coupling reaction.

Amine-containing dendrimers also have been used to quantify phosphorylated peptides for phosphoproteome analysis by mass spec (Tao *et al.*, 2005). In this novel application, proteins undergoing analysis are reduced and alkylated with iodoacetamide to block sulfhydryls, then trypsin digested. The peptides are then desalted and lyophilized. The dried peptides are methylated in methanolic HCl to eliminate interference by carboxylate groups and then the solvent is removed in vacuo. The mixture of blocked peptides next is reacted with a G-5 PAMAM dendrimer using 50 mM EDC and 100 mM imidazole in 0.2 M MES buffer, pH 6.0 (see Chapter 27, Section 2.1). The reaction activates the phosphoryl groups on the phosphorylated peptides, which in turn react with the amines on the dendrimers to create phosphoramidate linkages (Figure 7.17). The conjugated phosphopeptides can be separated from non-phosphorylated peptides by simple filtration and washing. Finally, the isolated phosphopeptides can be cleaved from the dendrimer using 10 percent TFA treatment and analyzed by mass spec.

Epoxy Activation of Amine-Dendrimers

Epoxy groups can be formed on the surface of amine-containing dendrimers by reaction with either bis-epoxide compounds or epibromohydrin (or epichlorohydrin). Modification with these compounds forms terminal epoxide groups that can be used for subsequent conjugation with amine-, thiol-, or hydroxyl-containing ligands. Singh (1998) described a simple procedure using epibromohydrin to form an epoxy-activated PAMAM dendrimer for conjugation with thiol-modified alkaline phosphatase enzymes (Figure 7.18).

The following protocol is based on the method of Singh (1998). Other amine-containing dendrimers besides the PAMAM type, such as the PrioStar dendrimers from Dendritic Nanotechnologies may be used as well.

Protocol

1. Dissolve a dendrimer in 50 percent methanol/water at a concentration of at least 10 mg/ml. Singh (1998) used about 165 mg of a G-5 PAMAM dendrimer dissolved in 1 ml of the alcohol/water mixture. Use a fume hood for all operations.
2. Add 200 mg of sodium carbonate per ml of the dendrimer solution prepared in step 1 and a quantity of epibromohydrin equal to a 285-fold molar excess over the amount of

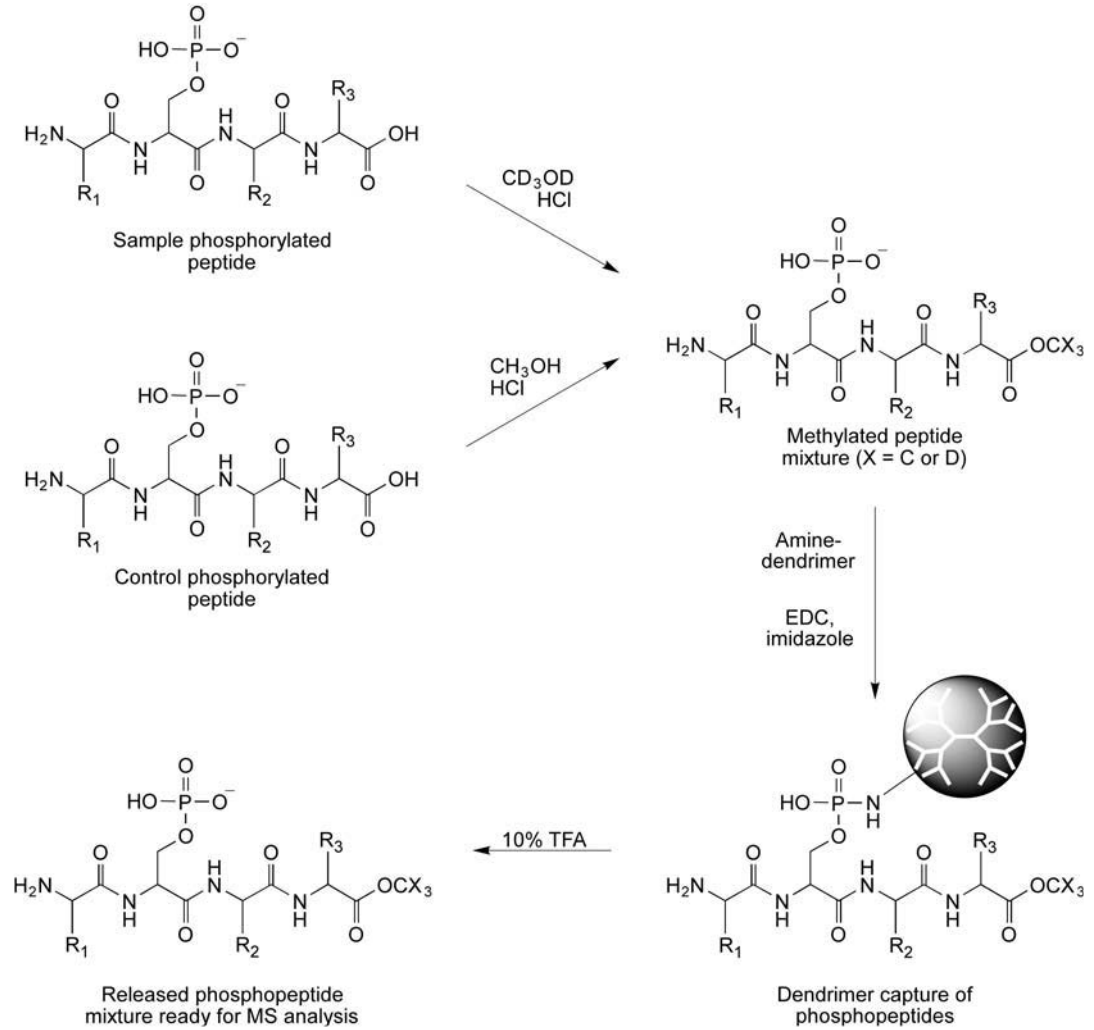


Figure 7.17 Phosphopeptides can be separated for mass spec analysis using an amine-dendrimer. A control and test sample first are both methylated to block their carboxylate groups and then mixed together in equal amounts. An amine-containing dendrimer then is used to capture the phosphorylated peptides by conjugation with the phospho groups using EDC and imidazole to form phosphoramidate bonds. After separation of the phosphopeptide-dendrimer conjugates using size exclusion chromatography, the phosphopeptides are cleaved off using TFA and analyzed by mass spec.

dendrimer present. The large excess of reagent assures that the amines get fully blocked during the reaction and will prevent any amines from crosslinking by reaction with the epoxy ends of the modified dendrimer.

3. React for 4 hours with mixing in a fume hood.
4. Dilute the reaction mixture 10-fold with 50 percent methanol/water and purify the epoxy-activated dendrimer by repeated ultrafiltration using a membrane with a molecular

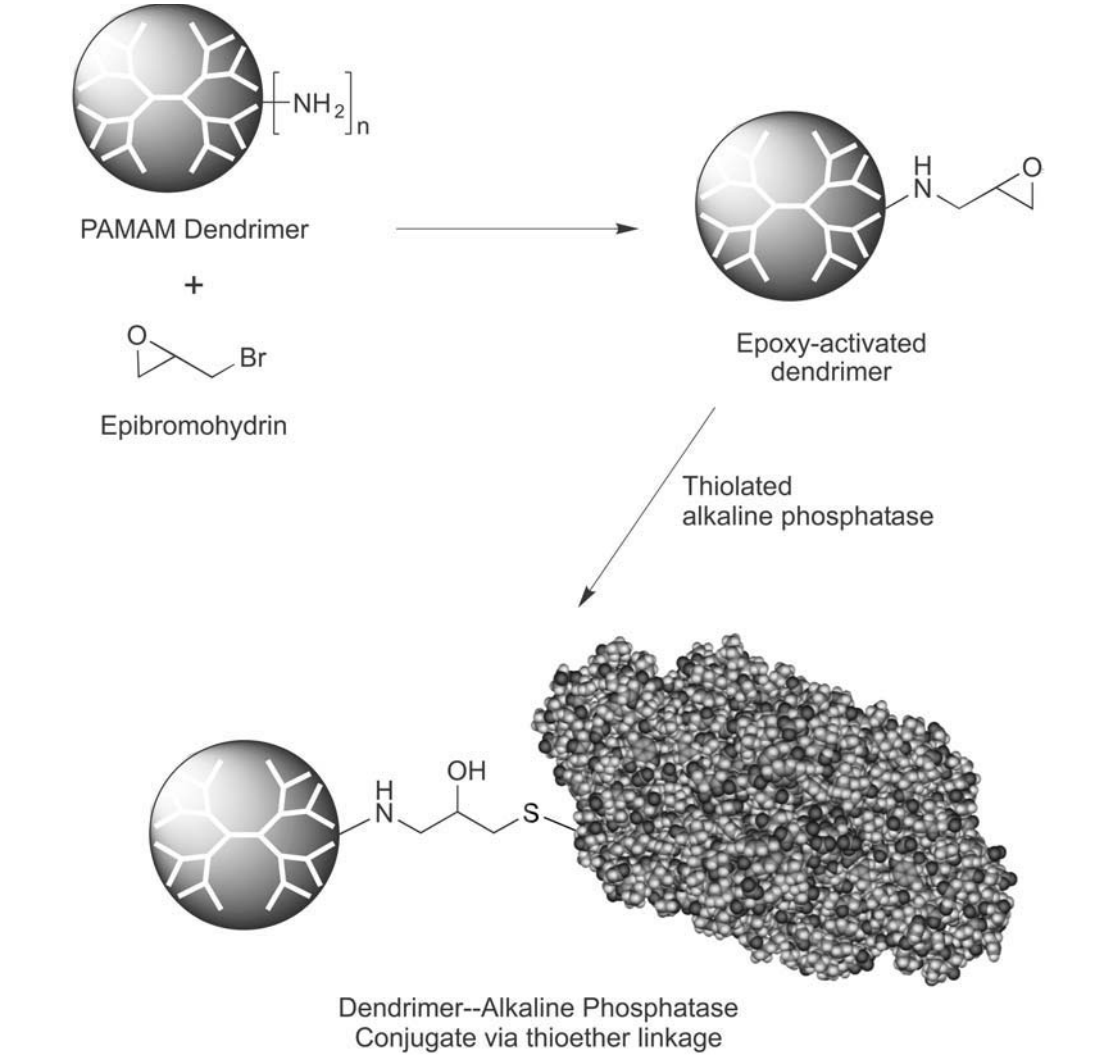


Figure 7.18 Amine-containing dendrimers can be activated with epibromohydrin to result in the formation of reactive epoxy groups on the dendrimer surface. This reactive intermediate then can be used to conjugate with thiol-containing proteins, such as thiolated alkaline phosphatase. The reaction results in the formation of a thioether bond.

weight cutoff able to retain the size of dendrimer being activated. Repeat the dilution and concentration steps until the filtrate is neutral in pH.

5. The epoxy-activated dendrimer may be conjugated to thiol-containing proteins by reaction in 50 mM sodium phosphate, pH 7.2. The reaction can be done at 4°C or at room temperature for 8–16 hours to form thioether linkages.
6. Purify the conjugate by gel filtration to separate protein-dendrimer complexes from excess protein or dendrimer.

Biotinylation of Amine-Dendrimers

Amine-containing dendrimers can be modified to contain one or more biotin groups for interaction with avidin or streptavidin reagents. The polyvalent nature of a dendrimer permits the formation of a biotin multimeric structure for potential enhancement of avidin–biotin assays. Since a biotin-dendrimer scaffold allows many biotin-binding proteins to dock simultaneously and form larger complexes, these conjugates can recruit more detection molecules to bind at the site of an analyte. This can have direct effect on the sensitivity of immunoassays, such as fluorescence detection, enzyme linked immunoadsorbent assays (ELISAs), and western blotting procedures.

This type of biotinylated dendrimer-based signal amplification technique has been done to increase the detection of genomic DNA in suspension arrays (Borucki *et al.*, 2005), wherein a DNA dendrimer was biotinylated multiple times and also modified to contain a targeting oligo sequence. The DNA dendrimer, which in that case was a construct consisting of partially hybridized oligonucleotide sequences branching out from a central core, was modified to contain as many as 850–900 biotin molecules on its surface along with at least one targeting oligonucleotide sequence. After genomic DNA was allowed to bind to a capture oligo on a fluorescence microparticle, the biotinylated dendrimer was added and it bound to the genomic DNA via hybridization with the targeting oligo. Finally, streptavidin- phycoerythrin detection conjugate was added and it then was bound to the biotin groups on the dendrimer (Figure 7.19). The resultant fluorescent signal was amplified beyond that possible using a simple biotinylated oligo directly.

A similar type of biotin-dendritic multimer also was used to boost sensitivity in DNA microarray detection by 100-fold over that obtainable using traditional avidin–biotin reagent systems (Stears, 2000; Striebel *et al.*, 2004). With this system, a polyvalent biotin dendrimer is able to bind many labeled avidin or streptavidin molecules, which may carry enzymes or fluorescent probes for assay detection. In addition, if the biotinylated dendrimer and the streptavidin detection agent is added at the same time, then at the site of a captured analyte, the biotin–dendrimer conjugates can form huge multi-dendrimer complexes wherein avidin or streptavidin detection reagents bridge between more than one dendrimer. Thus, the use of multivalent biotin–dendrimers can become universal enhancers of DNA hybridization assays or immunoassay procedures.

Another example of an immunoassay enhancement using biotinylated dendrimers involves a novel detection technique called carbonylmetallo-immunoassay (CMIA). This technology involves the use of an NHS-4-pentynoate-(dicobalt hexacarbonyl) transition metal chelate labeling reagent or (n^5-cyclopentadienyl)iron dicarbonyl (n^1-*N*-maleimidato) group, which can be detected using Fourier transform infrared spectroscopy (Salmain *et al.*, 1991, 1992; Varenne *et al.*, 1992, 1995; Philomin *et al.*, 1994; Vessières *et al.*, 1999). These compounds can be coupled to amine groups on PAMAM dendrimers to yield amide linkages. Salmain *et al.* (2002) developed a dendrimer-based signal enhancement method to increase the sensitivity of the IR detection chelate. A G-4 PAMAM dendrimer containing 64 primary amine groups was labeled with NHS-biotin and the iron chelate to yield a complex containing 45 chelate groups and 4 biotins (Figure 7.20). The ratios in the final dendrimer conjugate were of course dependent on the molar ratios in the initial reaction mixture and could be controlled through careful planning of the conjugation process.

Biotinylated dendrimers also have been used to develop targeting conjugates for therapeutic use in targeting cancer cells. Wilbur *et al.* (1998) studied several different PAMAM

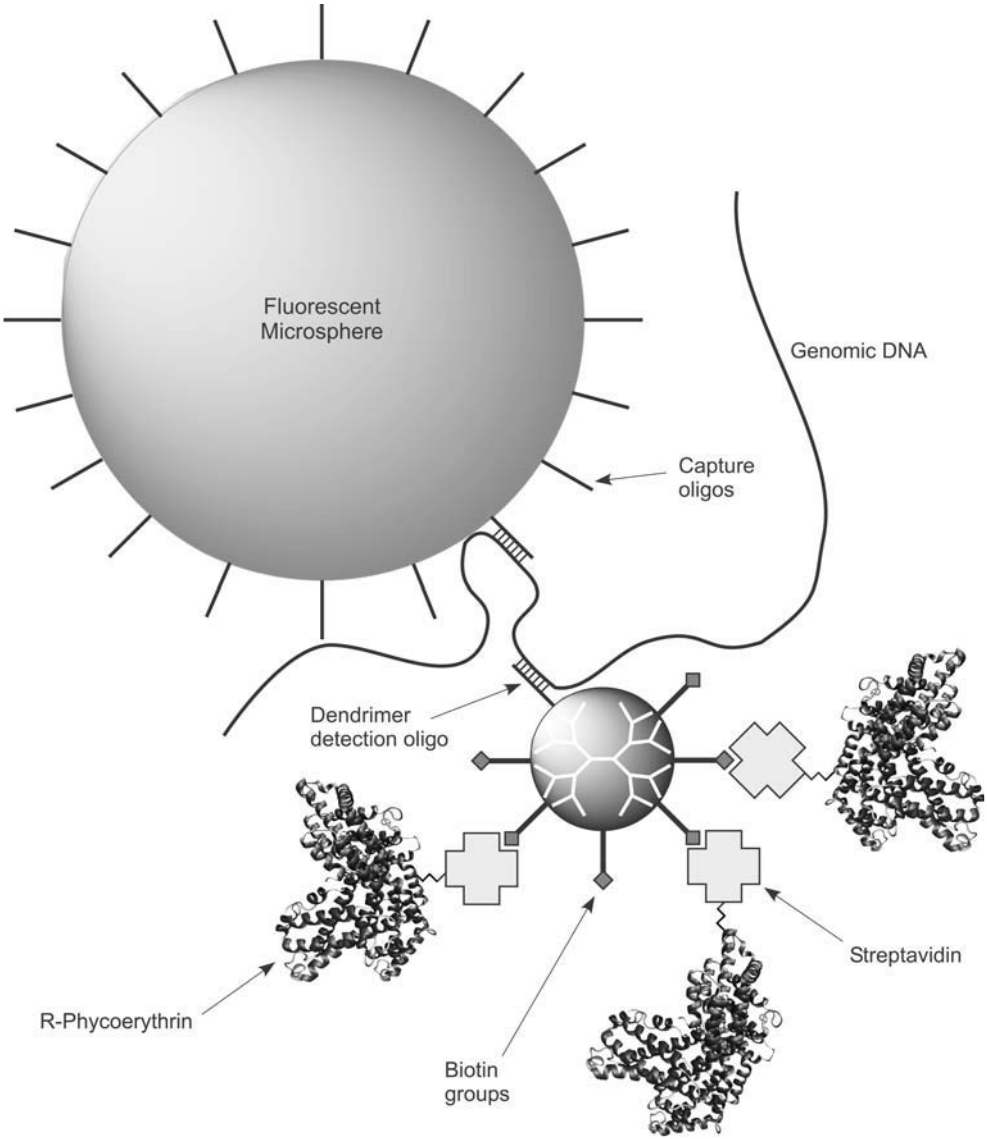


Figure 7.19 Biotinylated dendrimers can be used to enhance signals in assay procedures using the (strept)avidin–biotin interaction. In this example, a fluorescent microsphere containing capture oligos is able to interact with target DNA while a biotinylated dendrimer also containing detection oligo sequences binds to the genomic DNA target. The interaction of the dendrimer is detected using streptavidin–phycoerythrin conjugates, which are intensely fluorescent.

generations in the coupling of a water-soluble biotin analog with unique biotinidase insensitivity. The biotin-dendrimer conjugates were compared with biotin trimers or biotin tetramers in their ability to recruit radionuclide-labeled streptavidin complexes after binding to a streptavidin–antibody conjugate already bound to antigen on a tumor cell surface. It was found that the

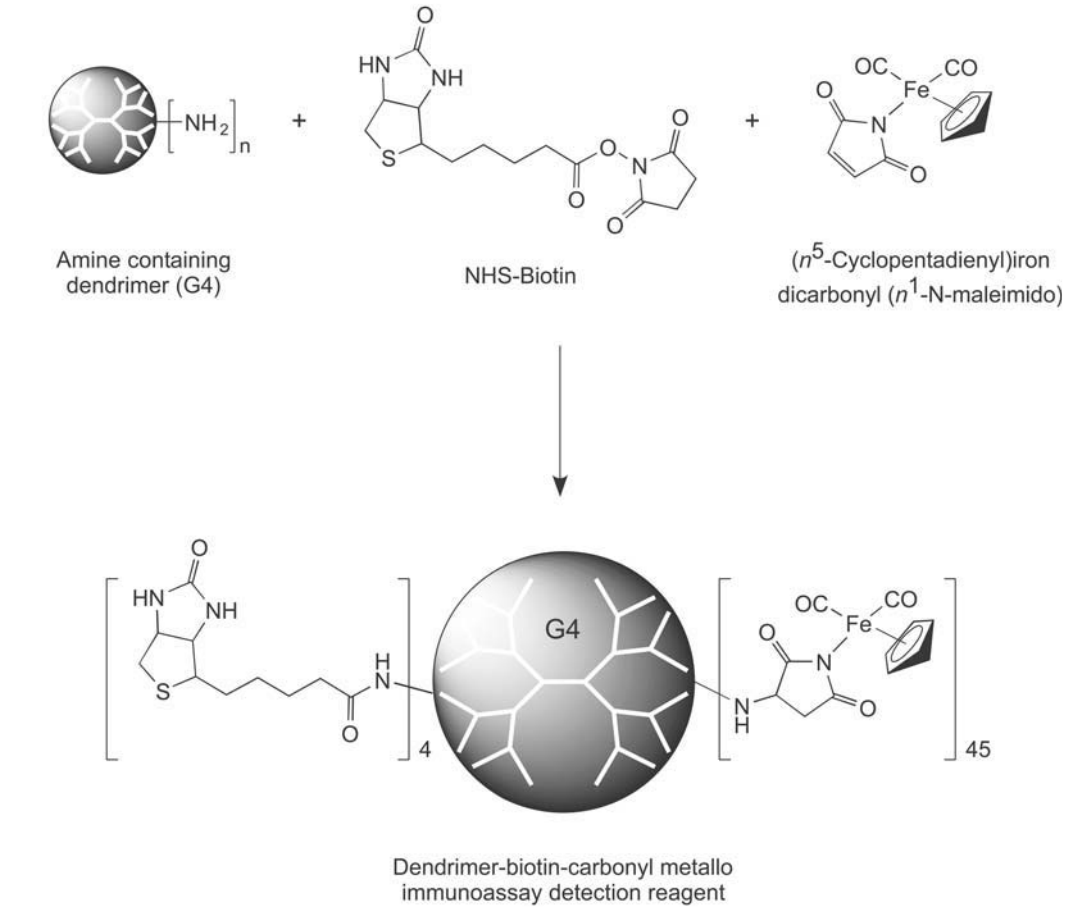


Figure 7.20 The multivalent surface of dendrimers can be used to couple biotin groups and labels for detection in immunoassays. One such conjugate was made by coupling NHS-biotin and a maleimido-iron chelate to an amine-dendrimer for use in an unique carbonyl metallo assay method.

biotinylated dendrimer increased the number of radionuclide-streptavidin binding events by a factor of 4.

The efficiency of biotinylation also was determined relative to the size of the amine-dendrimer (Wilbur *et al*., 1998). The reactions were done in DMF with triethylamine added as a proton acceptor, so competing hydrolysis of the NHS ester on the biotin compound was not an issue. In reacting each generation of dendrimer with a large excess of biotinylation agent, it was found that G-0, G-1, G-2, and G-3 PAMAM dendrimers all could be completely biotinylated by modification of all of the pendent amine groups. Only when reaching the size of a G-4 dendrimer was the biotinylation yield less than the number of total amine groups present (51 of 64 possible amines modified). This indicates that, unlike with protein labeling, the reactivity and accessibility of available amines on dendrimers remains high even when working with mid-sized molecules.

Mamede *et al*. (2003) developed a biotinylated G-4 PAMAM dendrimer containing multiple diethylenetriamine pentaacetic acid (DTPA) chelating groups for use in radiotherapy of intraperitoneally disseminated tumors. The G-4 dendrimer was reacted with sulfo-NHS-LC-biotin at a molar ratio that resulted in approximately 2.5 biotin molecules per dendrimer. This conjugate then was conjugated with 2-(*p*-isothiocyanatobenzyl)-6-methyldiethylenetriaminepentaacetic acid to give about 52 DTPA chelating groups per dendrimer. Finally, the complex was loaded with radioactive ^{111}In and also incubated with avidin to result in 2–3 avidin molecules per dendrimer binding to the biotin groups on the surface. The positive charge of the avidin protein molecules facilitated entry into the tumor cells, while the radioactive cargo killed the cells.

The biotinylation of amine-dendrimers may be accomplished using either an organic reaction environment or an aqueous medium. For modification of PAMAM dendrimers with a biotinidase resistant biotin compound, Wilbur *et al*. (1998) performed the reaction in DMF with triethylamine as catalyst (proton acceptor). The following protocol illustrates this type of procedure using the biotinylation reagent NHS-PEG_4-biotin, which closely compares to the biotinidase insensitive compound used in the published procedure.

Protocol

1. In a fume hood, dissolve an amine-containing dendrimer in DMF at a concentration of at least 10 mg/ml with mixing. For the modification of a G-3 dendrimer, Wilbur *et al*. (1998) used approximately 240 mg of dendrimer dissolved in 10 ml of DMF. Other concentrations will work well in this procedure.
2. Add 15.7 µl of triethylamine (0.12 mmol) to the dendrimer solution with mixing.
3. Dissolve a quantity of NHS-PEG_4-biotin (MW 588.67) in the dendrimer solution with mixing to bring the final reagent-to-dendrimer ratio in the reaction medium to at least 1.25 times greater than the molar amount of amines to be modified. To saturate completely a G-3 dendrimer initially containing 32 primary amine groups, add at least 8 mg of biotinylation reagent for each mg of dendrimer being modified. Using this molar ratio of biotin-to-dendrimer in the reaction will result in a high-modification yield of all the amine groups on the dendrimer, especially for G-1 through G-3 size dendrimers. If a lower modification level is desired, then scale back the amount of biotinylation reagent added accordingly. *Note*: The most important consideration is optimizing the molar ratio of biotinylation reagent to dendrimer to obtain the desired modification level in the final conjugate. This entirely depends on the intended application of the biotinylated dendrimer, and a series of reactions containing different biotinylation ratios may have to be done to determine the best ratio to use.
4. React for a minimum of 1 hour at room temperature with mixing. Longer reaction times may be used, particularly if a maximal modification level of biotin is desired.
5. Purify the biotinylated dendrimer by diluting it with an equal volume of water and then using dialysis, ultrafiltration, or size exclusion chromatography.

Another method that can be used to biotinylate an amine-dendrimer is to do a similar reaction in aqueous buffer conditions. The following protocol is based on the methods of Tomalia *et al*. (1998) and Mamede *et al*. (2003).

Protocol

1. Dissolve 10 mg (0.7 μmol) of a G-4 PAMAM dendrimer in 1 ml of 0.1 M sodium phosphate, pH 9.0.
2. Add a 3-fold molar excess of biotinylation reagent over the molar quantity of dendrimer present. For the use of sulfo-NHS-LC-biotin (MW 556), this represents the addition of 2.1 μmol or 1.16 mg. This reaction ratio will result in a modification level of about 2.5 biotin groups per dendrimer. Other molar ratios also may be used, depending on the desired level of modification and the intended use for the conjugate.
3. React for 30–60 minutes at room temperature with mixing.
4. Purify the biotin-dendrimer using size exclusion chromatography on a desalting matrix or by use of ultrafiltration (e.g., centrifugal concentrators).

Fluorescent Labeling of Amine-Dendrimers

The multivalent nature of dendrimers can be used to advantage for signal enhancement in many fluorescence detection schemes. A broad selection of hydrophilic organic fluorescent probes is available as amine-reactive derivatives (Chapter 9), many of which may be used to form conjugates with amine-containing dendrimers. Coupling multiple fluorescent labels to each dendrimer molecule creates a complex with increased luminescence or brightness for use in fluorescence assays (Tomalia *et al*., 1998). In addition, combining a fluorescently labeled dendrimer with the ability to link it to targeting molecules, such as antibodies or streptavidin, provides a reagent system having superior detection capability than the use of fluorescently labeled proteins directly. Manduchi *et al*. (2002) demonstrated that a fluorescent dendrimer could be a more sensitive detection reagent than the use of directly labeled primary antibodies or indirect immunoassay methods using labeled secondary antibodies for microarray assay methods.

For instance, a dendrimer easily can be coupled with a large number of fluorescent dyes and still provide additional coupling sites for biotinylation. The only limitation to the number of fluorescent modifications is if fluorescence quenching starts to take place, in which case no further modifications will result in increased signal. A series of such conjugates using different levels of fluorophore modification should be done to determine the optimal level of dye-to-dendrimer before quenching occurs.

A fluorescent, biotinylated dendrimer of this type then can be used in a (strept)avidin–biotin assay to detect analytes. Having more than one biotin group on such a conjugate allows it to interact with multiple streptavidin molecules, thus forming a megameric complex with a biotinylated primary antibody bound at the site of an analyte. In this way, many more fluorescent molecules are recruited to the point of detection than is possible using directly labeled proteins (Figure 7.21). In fact, for detection of DNA targets, dendrimer-based fluorescence enhancement methods have been identified as an important route to increasing signal and avoiding polymerase chain reaction (PCR) or other amplification methods (Nilsen *et al*., 1997; Vogelbacker *et al*., 1997; Kricka, 1999).

Fluorescent labels on dendrimers also are convenient to track the interaction of functionalized dendrimers for cell targeting. After the conjugation of targeting groups such as antibodies or folic acid on a dendrimer along with radiolabels or toxic components, there are still amine groups available to add fluorescent labels for detection purposes (Minard-Basquin *et al*., 2003; Patri *et al*., 2004; Majoros *et al*., 2005). Such complexes may be assessed as to their specificity by monitoring the fluorescent signal as the dendrimers bind to target cells.

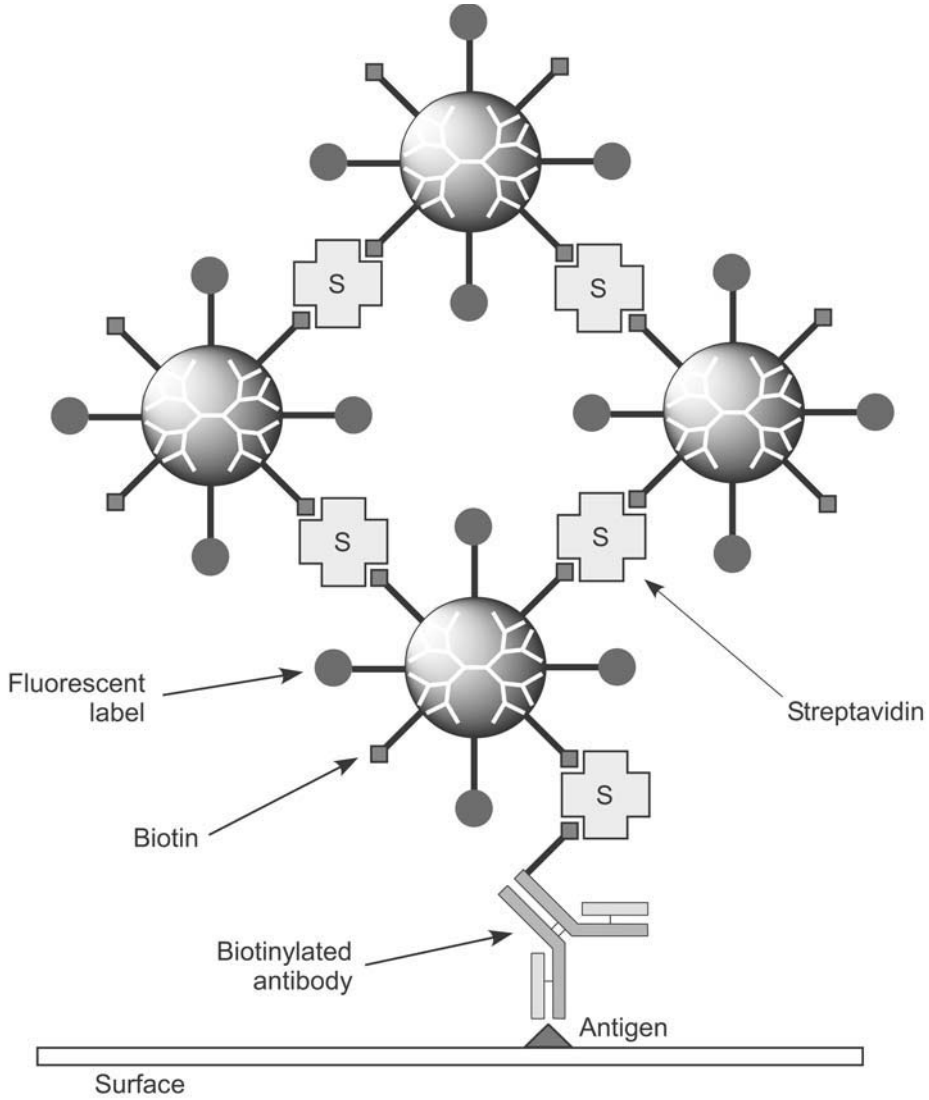


Figure 7.21 Dendrimers that are fluorescently labeled as well as biotinylated create enhanced detection reagents for use in (strept)avidin–biotin-based assays. Large complexes containing multiple fluorescent dendrimers can bind to antigens and form a highly sensitive detection system that exceeds the detection capability of fluorescently labeled antibodies.

Dendrimers bearing certain fluorescent molecules attached on their periphery have been shown to be environmentally sensitive probes for the presence of certain metal ions or to changes in pH (Balzani *et al*., 2000; Paola *et al*., 2005). In addition, PAMAM dendrimers modified with the relatively hydrophobic dye Oregon Green 488 were shown to be a more effective transfection agent for anti-sense oligonucleotides than the dendrimer alone–plus the complex could be tracked within the cell due to the fluorescence of the dye (Yoo and Juliano, 2000).

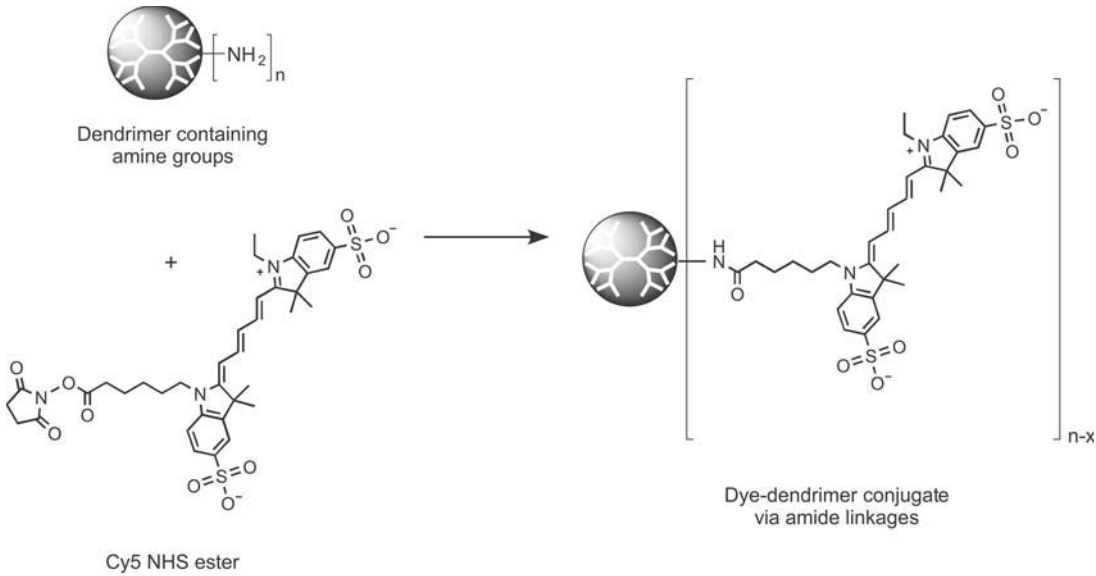


Figure 7.22 Fluorescent dyes such as an amine-reactive Cy5 derivative can be coupled to amine-dendrimers at relatively high substitution levels to create intensely fluorescent detection agents. If the dendrimer also is derivatized to contain an affinity group or a targeting group then specific fluorescent detection at high sensitivity can be realized.

Specific procedures for the conjugation of fluorescent labels to amine-containing molecules can be found in Chapter 9. The following protocol describes one such reaction for an amine-dendrimer with the fluorescent cyanine reactive probe, DyLight 649 NHS ester. This dye has spectral properties that are nearly identical to the original Cy5-type dye, but the DyLight one has negatively charged sulfonate groups, which makes the resultant compound extremely water-soluble. Coupling reactions with other fluorescent dyes may be done similarly, but in each case, optimization of the molar ratio of dye-to-dendrimer should be done to produce the best conjugate for its intended application. Note that higher levels of dye incorporation do not necessarily correlate to brighter fluorescent conjugates, because fluorescence energy transfer and quenching may occur between dye molecules as the density of dyes increases on the dendrimer surface. However, it appears that fluorescent dye substitution on dendrimers can be done at a higher density than on proteins before such quenching effects occurs. Figure 7.22 illustrates the reaction between an amine-dendrimer and a disulfonated Cy5 NHS ester derivative. Although the exact structure of the DyLight 649 NHS ester is not published, it is similar to the known Cy5 structure, but containing additional sulfonate groups to increase water solubility.

Protocol

1. In a fume hood, dissolve the DyLight 649 NHS ester at a concentration of 10 mg/ml in dry DMF.
2. Dissolve the amine-dendrimer to be modified in DMF or buffer (50 mM sodium borate, pH 8.5) at a concentration of at least 10 mg/ml. Avoid the use of amine-containing buffers for an aqueous reaction, such as Tris or imidazole, as these will react with the

NHS ester on the dye and prevent conjugation to the dendrimer. For reactions done in organic solvent, add triethylamine to a final concentration of 1.25 times greater than the amount of reactive dye to be added to the solution.

3. Add a quantity of the DyLight 649 dye to the dendrimer solution to provide at least a 1.25-fold molar excess of dye over the amount of dendrimer present (for nonaqueous reactions) or a 6–15-fold molar excess for aqueous reactions. Mix well to dissolve. The optimal amount of dye added should be determined experimentally by preparing a series of conjugates using different molar ratios of dye-to-dendrimer.
4. React for 1 hour at room temperature with mixing.
5. Remove unreacted dye and reaction by-products from the modified dendrimer by gel filtration or dialysis, using a molecular weight cutoff suitable for the size of the dendrimer.

3. Dendrimer-Chelate Derivatives for Imaging Applications

Imaging agents consisting of metal chelating compounds have been used extensively for the modification of targeting agents for the *in vivo* detection of specific cells, organs, or vascular systems. The proper chelating group can coordinate securely a radioactive element or a contrast-enhancing agent, which can be imaged using radio-imaging techniques or magnetic resonance imaging (MRI), respectively. Many such poly-dentate chelating agents are described in Chapter 10. These compounds are designed to have bifunctional characteristics in that one end is reactive for coupling to another molecule and the other end contains the chelating group.

The polyvalent nature of dendrimers has been investigated as vehicles for carrying multiple chelator groups to enhance signals in various imaging applications (Barthand Soloway, 1994; Yoo *et al*., 1999; Kobayashi *et al*., 2000, 2001; Sato *et al*., 2001). In addition, in certain chelate-dendrimer constructs, excess amines on the dendrimer surface can aid in the cellular uptake process through charge-mediated endocytosis.

Roberts *et al*. (1990) conjugated porphyrins to PAMAM dendrimers as carriers to link them to antibody molecules for *in vivo* targeting. The porphyrin derivative was a *N*-(4-nitrobenzyl)-5-(4-carboxylphenyl)-10,15,20-tris(4-sulfophenyl)porphine containing one carboxylate group, which could be coupled to the amine groups on the dendrimer using the EDC/NHS method to form an amide bond. This porphyrin compound was effective at chelating copper ions, especially ^{67}Cu, which has nuclear decay properties ideal for potential use as a chemotherapeutic agent.

One of the more common chelating groups used for imaging applications is DTPA. This compound is available in amine-reactive form as the anhydride or as a isothiocyanatobenzyl derivative [e.g., 2-(*p*-isothiocyanatobenzyl)-6-methyldiethylenetriaminepentaaceticacid chelate (1B4M; MW 555) (Kobayashi *et al*., 1999). There are various isothiocyanatobenzyl-DTPA derivatives available that avoid conjugation of one of the chelator's carboxylate groups, which would be necessary using the anhydride form (Mirzadeh *et al*., 1990; Brechbiel and Gansow, 1991; Michel *et al*., 2002; Behr *et al*., 1999; Brouwers *et al*., 2003; Macrocyclics, Inc.). Any of these derivatives can be used to modify an amine-dendrimer to contain multiple chelating groups on its surface (Figure 7.23). Mamede *et al*. (2003) used the SCN-Bzl-DTPA compound 1B4M to modify a biotinylated, G-4 PAMAM dendrimer to contain approximately 52 chelating group substitutions. The conjugate then was loaded with ^{111}In and complexed with avidin for investigation of its use for radiotherapy applications.

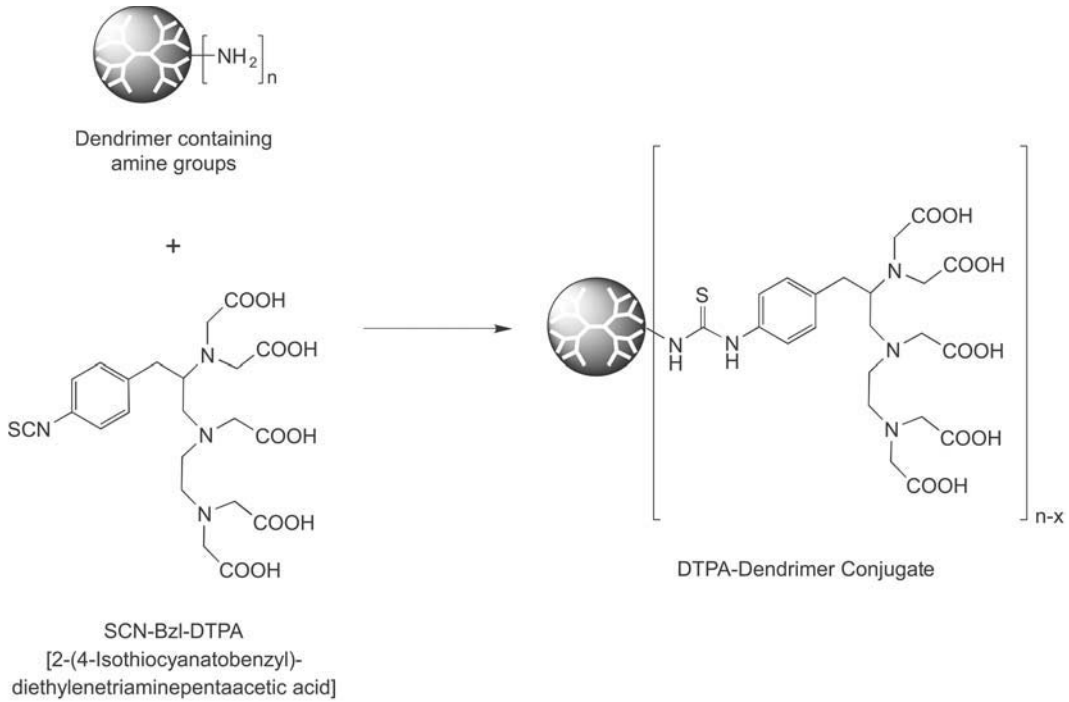


Figure 7.23 Chelating groups such as the isothiocyanate derivative of DTPA can be used to create multivalent chelating complexes with amine-dendrimers. Such complexes are able to coordinate multiple metal ions for detection, imaging, or radioimmunotherapy purposes.

Kobayashi *et al.* (2003) similarly prepared a DTPA modified PAMAM dendrimer using the same isothiocyanate derivative for use in MRI imaging. A G-8 amine-dendrimer was reacted with a 1,024-fold excess of chelating compound, resulting in a heavily loaded complex. In this case, the resultant conjugate was charged with ^{153}Gd to create a high-intensity contrast agent for *in vivo* imaging use in animals.

The following protocol for the modification of an amine-dendrimer with an SCN-Bzl-DTPA chelator is based on the literature references previously cited. Dendrimers of other generations will work well in this procedure provided that the molar ratios of reactants are adjusted for the size of dendrimer being used and the substitution level desired.

Protocol

1. Dissolve 10 mg of a G-4 amine-dendrimer in 1 ml of 0.1 M sodium carbonate buffer, pH 9.0.
2. Add a quantity of the SCN-Bzl-DTPA bifunctional chelating agent to obtain the desired molar excess of label over the amount of dendrimer present. The optimal ratio may be determined experimentally by preparing a series of dendrimer-chelate conjugates using different molar ratios and choosing the one that works the best in the intended application.

3. React for at least 1 hour at room temperature with mixing. Some reactions have been done for up to 24 hours at elevated temperatures (e.g., 40°C).
4. Purify the DTPA-dendrimer using dialysis, size exclusion chromatography, or spin-tube concentrators having a molecular weight cutoff of 5,000 Daltons.

Other chelate-dendrimer constructs may be prepared using alternative bifunctional chelating agents according to Chapter 10.

4. Dendrimer Derivatives as Surface Modification Agents

The extremely branched nature of dendrimers provides multiple functionalities for creating high-capacity surfaces for coupling affinity molecules. The presence of numerous pendent groups on dendrimers can be used to advantage both in facilitating attachment to the surface and in providing a biocompatible coating for protein coupling. For instance, dendrimer modification of gold surfaces, both planer and nanoparticle, have the potential to create a matrix for high-density ligand coupling and low nonspecificity in assays. However, the dendrimer type and the characteristics of the pendent functionalities come into play in forming the optimal surface environment. PAMAM or amine-dendrimers of other types could result in relatively high nonspecific binding, particularly if unmasked terminal amines provide sites for charge interactions with other proteins (Yoon *et al*., 2002; Hong *et al*., 2003a, 2004;).

Amine-dendrimers also can be used to pattern biological molecules on gold surfaces (Hong *et al*., 2003b). In this application, PAMAM dendrimers were printed onto a SAM surface prepared using 11-mercaptoundecanoic acid to create sites for linking biomolecules. The carboxylate groups on the termini of the decanoic acid groups were activated with carbodiimide (EDC) and petafluorophenol to create reactive PFP esters (Chapter 2, Section 1.13). A PAMAM dendrimer solution in methanol (0.7 nM) was printed onto this activated SAM surface by contact printing. Reaction between the PFP esters on the SAM molecules and the amines on the PAMAM molecules covalently coupled the dendrimers to the surface via amide bond linkages. The dendrimers then were biotinylated with sulfo-NHS-biotin, washed, and allowed to interact with fluorescently labeled avidin, which was bound to the surface through interaction with the biotin groups. The fluorescent label was used to detect the bound protein and assess the efficiency of dendrimer surface functionalization.

Alternatively, PAMAM dendrimers can be linked to glass surfaces containing aldehyde functionalities through Schiff base formation and reductive amination using sodium cyanoborohydride. Hong *et al*. (2003b) patterned dendrimers on aldehyde slides and then blocked excess aldehyde groups using a 2 hour incubation with 1 M 2-(2-aminoethoxy)ethanol. The result was covalently linked dendrimers on the slides containing an abundance of dendritic amines for further conjugation.

Hong *et al*. (2004) also found that modification of PAMAM dendrimers with a short PEG linker arm could act to reduce nonspecificity caused by the amines on the dendrimer-modified surface. An azido-PEG_3-amine spacer was activated with nitrophenyl carbamate to yield an activated intermediate that could be used to modify the amines on the dendrimer (Figure 7.24). Reaction at high molar ratio resulted in about 61 PEG-azido spacers on the dendrimer. Reduction of the azido group to an amine using triphenylphosphine in THF provided the dendrimer-PEG-amine derivative for surface modification. The added presence of the PEG spacer arm reduced

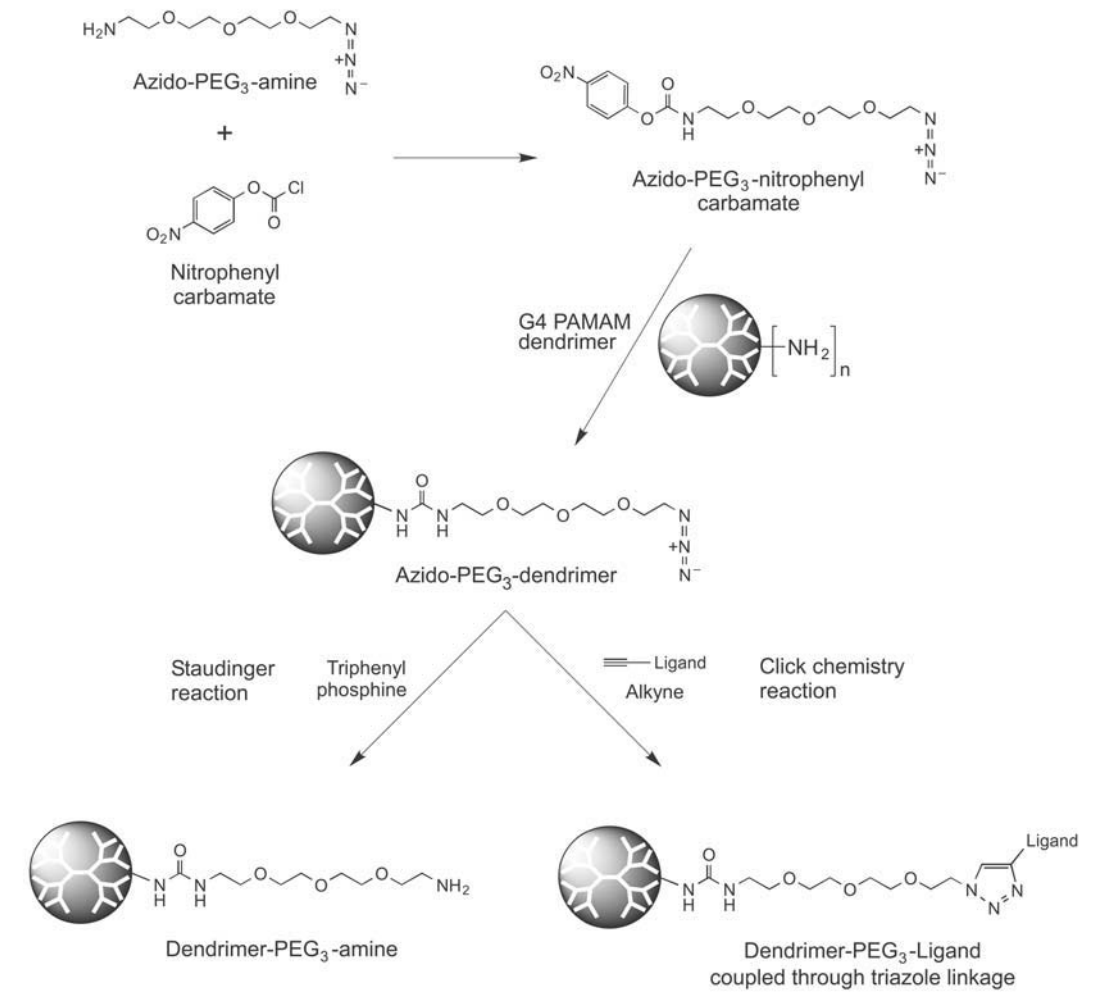


Figure 7.24 Azido-PEG-amine linkers can be coupled to amine-dendrimers by formation of an intermediate reactive nitrophenyl carbamate group. This compound then can be reacted with the amines on a dendrimer to form an azido-dendrimer derivative that can be used in chemoselective ligation strategies. The azide groups can be reacted with alkyne derivatives in a click chemistry reaction to couple ligands through a triazole linkage. Alternatively, the azido groups can undergo a Staudinger reaction with a phosphine compound to give the amino-PEG-dendrimer derivative. In addition, a Staudinger ligation reaction can be done using a phosphine derivative that would result in a covalent amide bond linkage (see Chapter 17, Section 4).

nonspecific binding to the dendrimer-surface despite the fact that both the PAMAM and PEG-modified dendrimer both had terminal amino groups present. Another bioconjugation route to using this azido-PEG-dendrimer is to make an alkyne-containing protein or ligand and couple it to the modified dendrimer using click chemistry. The reaction of an azide with an alkyne in the presence of Cu(I) results in a cycloaddition reaction, which forms a triazole ring. See Chapter 17, Section 4 for more details on the click reaction.

In another interesting route to creating dendrimer-functionalized surfaces, Pathak *et al.* (2004) activated the silanol groups on glass slides using CDI (Chapter 3, Section 3). The resultant reactive imidazole carbamate groups could be coupled to amine-containing dendrimers to form covalent carbamate linkages directly to the surface. Studying the effect of surface modification with generations 1–5 of a series of poly(propyleneimine) dendrimers resulted in the conclusion that dendrimers of G-3 size and above created surfaces of very high-binding activity for coupling affinity ligands, with a G-4 dendrimer giving the greatest binding potential. This method may provide an excellent modification strategy for immobilizing affinity ligands onto planer and spherical silica surfaces. Once the amine-dendrimer surface was created, coupling proteins to it was done using the heterobifunctional crosslinker, sulfo-GMBS, which formed a thiol-reactive surface.

Benters *et al.* (2002) used three different coupling strategies to coat glass slides with amine-dendrimers for subsequent immobilization of oligonucleotides for preparing DNA microarrays. Cleaned slides were initially modified with 3-aminopropyltriethoxysilane (APTS) or glycidyloxypropyltrimethoxysilane (GOPTS) to create functional groups for further modification. The APTS modified slides subsequently were reacted either with glutaric anhydride to form terminal carboxylate groups or with 1,4-phenylenediisothiocyanate (PDITC) to create reactive isothiocyanate groups on the surface. The carboxylate-containing slides were activated using the carbodiimide DCC along with NHS in DMF to form reactive NHS ester groups for coupling the amine-dendrimers.

G-4 PAMAM dendrimers were attached to each of the three activated slides using a 100 mg/ml solution in methanol. The result formed covalently attached dendrimer layers on the slide surfaces, which then were again activated for the coupling of oligonucleotides. The dendrimer activation process was similar to that of the APTS surface to form reactive NHS esters. The dendrimers first were modified with glutaric anhydride and then activated using DCC and NHS in DMF. This process formed amine reactive NHS esters on the dendrimers, which then could be used to immobilize directly 5′-amine modified oligos (Figure 7.25).

Dendrimer coated slides prepared using the methods of Benters *et al.* (2002) were found to provide significantly greater signal in fluorescent DNA hybridization assays than conventional slides prepared by coupling DNA directly to silane or poly-L-lysine modified surfaces. Clearly, dendrimers provide a three-dimensional surface with greater functionality and biocompatibility for producing high-activity arrays.

Le Berre *et al.* (2003) also investigated the use of dendrimers to provide enhanced coupling capability and increased signal for DNA microarrays. In this application, the dendrimers consisted of a core of hexachlorocyclo-triphosphazene ($N_3P_3Cl_6$), which terminated in aryl aldehyde groups on the surface (Launay *et al.*, 1994, 1997; Slomkowski *et al.*, 1999). A G-4 aldehyde dendrimer containing 96 pendent aldehydes was coupled to a slide surface after modification with APTS to contain amines. Multiple Schiff base interactions with the amine groups on the derivatized slide effectively immobilized the dendrimers. Next, the spotting of amine-modified DNA to the aldehyde-dendrimer surface with overnight incubation followed by reduction with sodium cyanoborohydride resulted in covalently linking both the dendrimers to the surface and the oligos to the aldehyde groups through secondary amine bonds. A comparison of the dendrimer-coupled DNA slides to 11 commercially available activated glass slides indicated that the dendrimer slide provided among the highest fluorescence intensities in hybridization assays and 10- to 100-fold higher detection sensitivity than conventional slides.

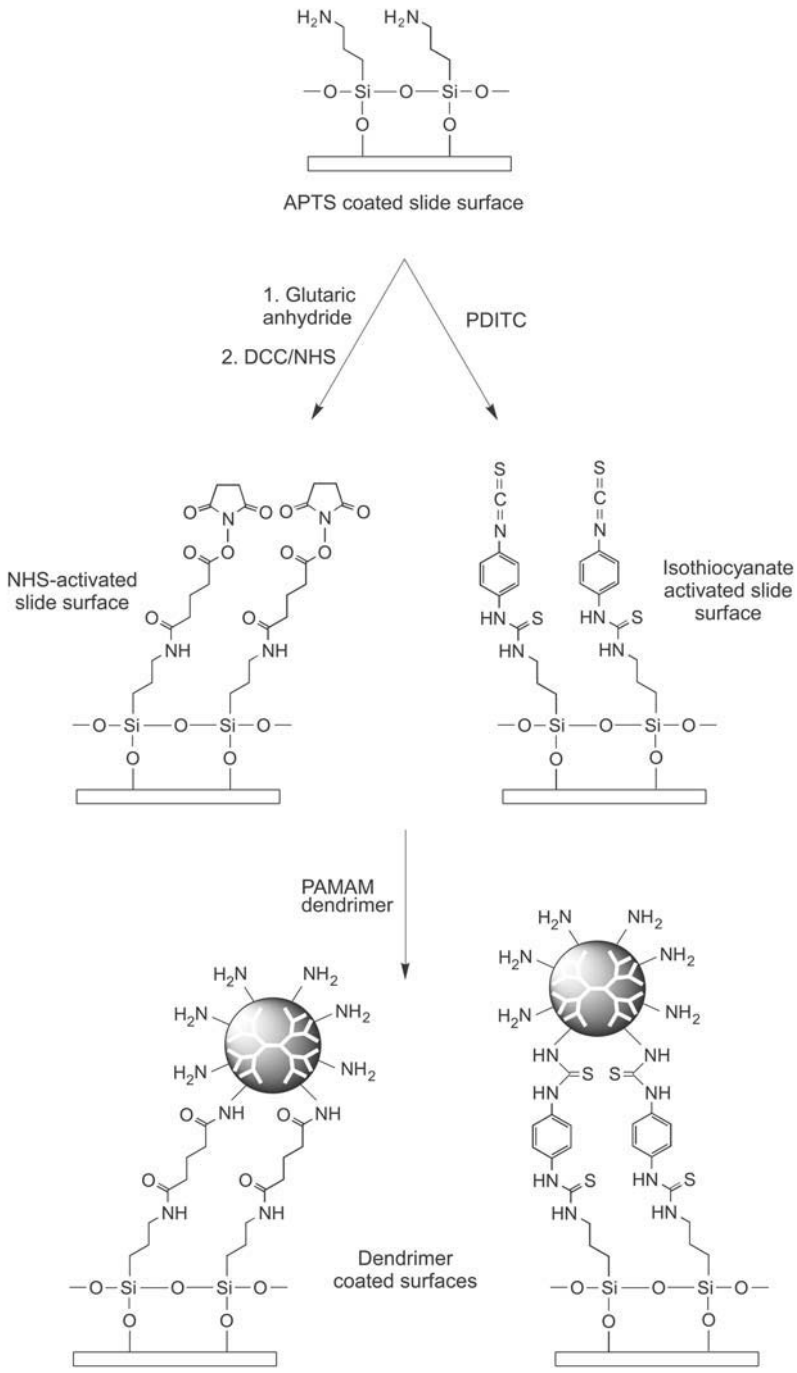


Figure 7.25 The multivalent nature of dendrimers can be used to add increased functionality to surfaces. Aminopropyl silane surfaces can be activated with either PDITC or through use of a cyclic anhydride plus DCC/NHS to give amine-reactive surfaces. These reactive surfaces can be used to couple amine-dendrimers to provide a high density of amine groups on the surface for further bioconjugation.

5. Dendrimer Fluorescent Quantum Dots

Dendrimers can be used to effectively coat and passivate fluorescent quantum dots to make biocompatible surfaces for coupling proteins or other biomolecules. In addition, the ability of dendrimers to contain guest molecules within their three-dimensional structure also has led to the creation of dendrimer-metal nanoclusters having fluorescent properties. In both applications, dendrimers are used to envelop metal or semiconductor nanoparticles that possess fluorescent properties useful for biological detection.

Huang and Tomalia (2005) used PAMAM dendrimers to coat gold nanoparticles or CdSe/CdS core/shell fluorescent quantum dots by preparing a disulfide-core dendrimer (using cystamine). The dendrimer then was succinylated to create terminal carboxylate groups, its core reduced with DTT, and the thiol-dendron used to modify quantum dots by dative bonding to the particle surface. The result was an organized polymeric coating on the gold particles or quantum dots that terminated in multiple carboxylate groups for conjugation of biomolecules (Figure 7.26). The negative charges on the dendron terminals provided charge repulsion to maintain colloidal stability of the small nanoparticles in solution, while the polyvalent nature of the dendrons made available an abundance of coupling sites for conjugation.

Testing of G-1, G-2, and G-3 dendrimers in this application provided insight into the density of surface modification needed to passivate completely the particles and prevent aggregation. The G-1 dendron was insufficient in this regard, but both the G-2 and G-3 dendron were big enough to create a surface barrier, which resulted in excellent colloidal stability of the particles in solution.

Zheng and Dickson (2002) created a new type of fluorescent dendrimer construct by sequestering small nanoclusters of silver within hydroxyl-terminated G-2 or G-4 PAMAM dendrimers (16 hydroxyls on G-2 and 64 hydroxyls on G-4). The internal structure of a dendrimer is known to interact with charged silver ions in solution (Varnavski *et al.*, 2001). Without adding a reducing agent, such as sodium borohydride, which is typically used to form silver nanoparticles, it was discovered that the silver ion dendrimer complex could be photoactivated to cause silver reduction to elemental silver within the internal structure of the dendrimers. The resulting very small dendrimer/silver nanoclusters displayed strong fluorescence with absorption bands at 345 nm and 430 nm and a broad emission curve extending from slightly less than 500 nm to nearly 700 nm. By contrast, if borohydride reduction was used, larger silver nanoparticles were formed (3–7 nm) within the dendrimers, which displayed no fluorescence characteristics, but only strong plasmon absorption at 398 nm. Thus, small fluorescent nanoclusters containing only up to about 8 atoms of silver were formed by photoactivation and stably sequestered within the G-2 or G-4 interior. These properties were in agreement with studies on silver clusters of 2–8 atoms, which have been shown to have size-dependent fluorescence characteristics (Tani and Murofushi, 1994).

The broad emission band displayed by these silver/dendrimer constructs actually was found to consist of 5 overlapping fluorescent peaks caused by individual silver/dendrimer complexes. Each of these complexes evidently contained a uniquely sized silver nanocluster, which resulted in an individual emission peak. Therefore, all the silver/dendrimer complexes together in solution presented a combined average of these 5 discrete emission peaks, and thus displayed the broad emission band covering nearly 200 nm in width across the spectrum.

Lesniak *et al.* (2005) also describe the preparation and use of similar dendrimer/silver nanoclusters using G-5 PAMAM dendrimers terminated with either amino, hydroxyl, or

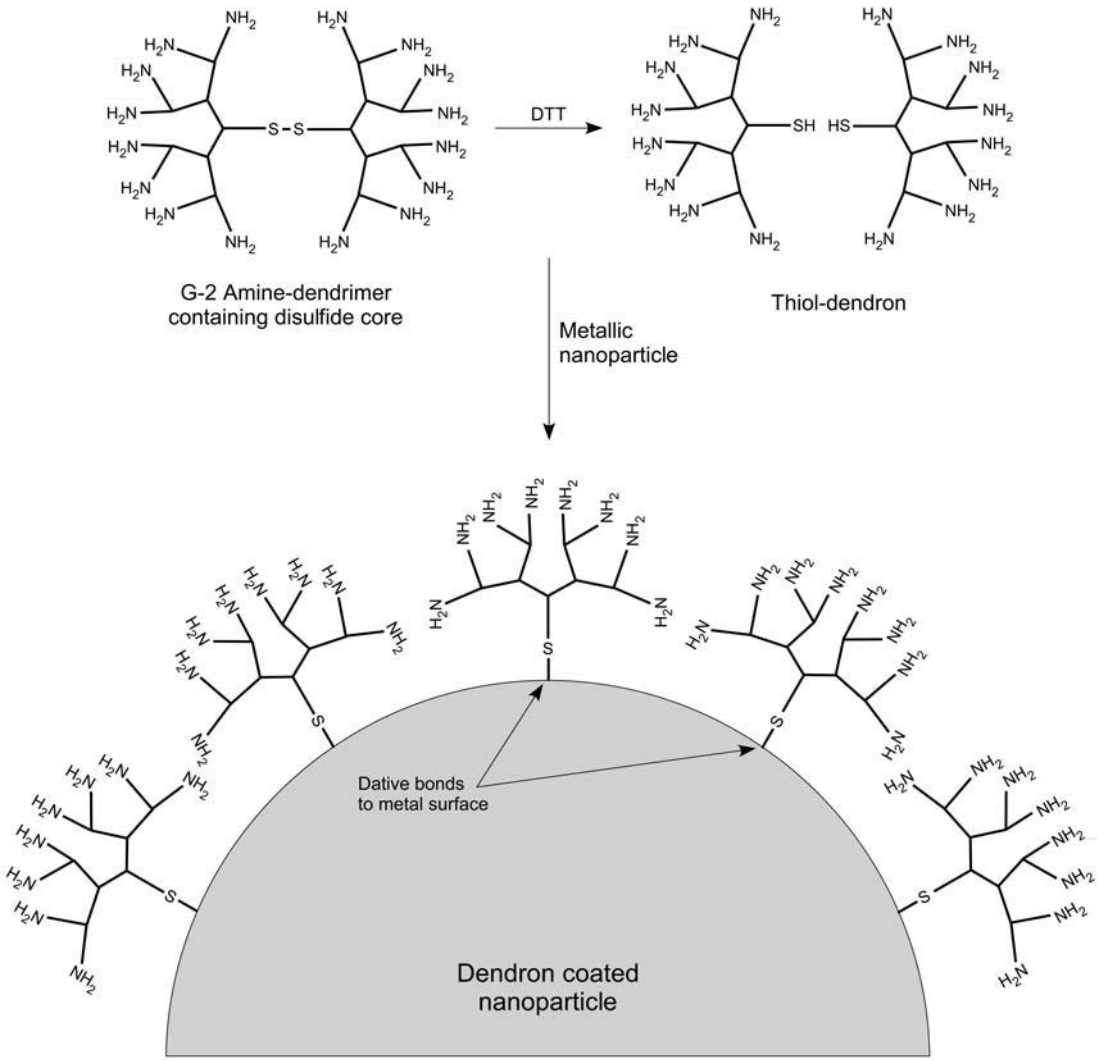


Figure 7.26 Dendrimers made with a disulfide-containing core can be reduced to produce dendrons having free thiol groups for surface modification. Dative binding of these thiol-dendrons to gold or metallic surfaces can provide a high density of amine groups for coupling proteins or other molecules.

carboxyl functionalities. In this method, 25 Ag (I) ions were entrapped within each dendrimer and photoactivated by irradiation with UV light. This reduced the silver ions to Ag (0) metal nanoclusters totaling 25 atoms per dendrimer. The resultant silver/dendrimer nanocomposites were fluorescent with excitation in the range of 300–400 nm and emission in the range of 400–500 nm. Investigations into the use of the complexes as fluorescent detection reagents for cell-based imaging proved positive, with the properties closely matching the expected characteristics of the parent dendrimer for cell uptake and cytotoxicity.

8

Cleavable Reagent Systems

Crosslinking and modification reagents may possess functionality other than their reactivity toward certain chemical groups. In particular, the cross-bridge of the molecule can be designed to contain constituents that allow cleavage of the reagent after use. Occasionally, the coupling reaction itself provides linkages susceptible to subsequent cleavage. Why would you want to break a conjugate apart after having formed it? The ability can be very important in studies involving the biospecific interactions between two molecules, especially if only one of the molecules is known or characterized. Cleavability allows the conjugation reaction to be verified through identification of the crosslinked molecules after conjugation and purification of the complex. Precise points of modification can be determined by mass spec analysis after cleavage. In some cases, purification of unknown target molecules is facilitated by the ability to cleave the crosslinking bonds after isolation of the complex. For instance, a protein modified near its binding site with a photoreactive heterobifunctional crosslinker can be incubated with its receptor or specific binding molecule, a covalent linkage then can be formed by exposure to UV light, and the complex analyzed by subsequent cleavage of the crosslink.

In another example, ligands can be biotinylated with a cleavable biotinylation reagent and then incubated with receptor molecules. The resulting complex can be isolated by affinity chromatography on immobilized (strept)avidin. Final purification of the ligand–receptor can be accomplished by cleaving the biotin modification sites while the complex is still bound to the support. The receptor complex thus can be eluted from the column without the usual harsh conditions required to break the avidin–biotin interaction.

The ability to cleave a crosslink also can provide a means of transferring a label from one protein to another. For instance, a photoreactive heterobifunctional crosslinker that is iodinatable and cleavable can be used to tag an unknown receptor molecule after conjugation. For example, the crosslinker SASD (Chapter 5, Section 3.2) can be iodinated before it is employed in a crosslink ing reaction. It is then used to modify a protein through its amine-reactive NHS ester end and purified from excess crosslinker. After incubation of the modified protein with specific binding molecules (e.g., other proteins) and photoreactive crosslinking, the conjugate can be broken by reduction of the disulfide group within the cross-bridge of the reagent. Since the radiolabeled part of the crosslinker now is attached to the unknown interacting molecule, the tracer is effectively transferred from the initially modified molecule. Thus, unknown interacting molecules can be tracked after their binding to an SASD-labeled substance.

Another crosslinker, SAED (Chapter 5, Section 3.9), can be used in a similar fashion, but instead of transferring a radioactive label, it contains a fluorescent portion that is transferred to a binding molecule after cleavage. Similarly, sulfo-SBED routinely is used to study protein interaction. Cleavage of a disulfide bridge after capture of interacting proteins results in transfer of a biotin label to the unknown prey protein (Chapter 28, Section 3.1). The biotin modification then can be used to detect or isolate the unknown interactor for subsequent identification.

The ability to break a crosslink can be an important feature of a modification or conjugation reagent. This chemistry typically is built into the cross-bridge or reactive ends of a reagent using disulfides, glycol groups, diazo bonds, esters, sulfone groups, or acetal linkages. The following sections describe these chemical characteristics and their respective cleavage conditions.

1. Cleavage of Disulfides by Reduction

The formation of a disulfide linkage between crosslinked molecules is an important option for many conjugation chemistries. Examples of reagents that have this capability include the pyridyl disulfide containing heterobifunctionals like SPDP (Chapter 5, Section 1.1) and SMPT (Chapter 5, Section 1.2). Other non-sulfhydryl-reactive crosslinkers still may possess a disulfide group within their cross-bridge construction. The presence of such disulfide groups, whether designed in the crosslinker or created as a product of their reactions, allows for specific cleavage of the complex or modified molecule after conjugation. Disulfide bonds can be broken by a number of methods (Chapter 1, Section 4.1), utilizing either direct hydrogenolysis by a strong reductant such as sodium borohydride or through a disulfide interchange process with a compound containing one or more free sulfhydryls (Figure 8.1).

Cleavage of disulfide bonds is done easily by incubation with a reducing agent at a level of 10–100 mM concentration. If the disulfides in the crosslinks are the only ones present in the complexed molecules, then reduction will yield unconjugated molecules—one or both of

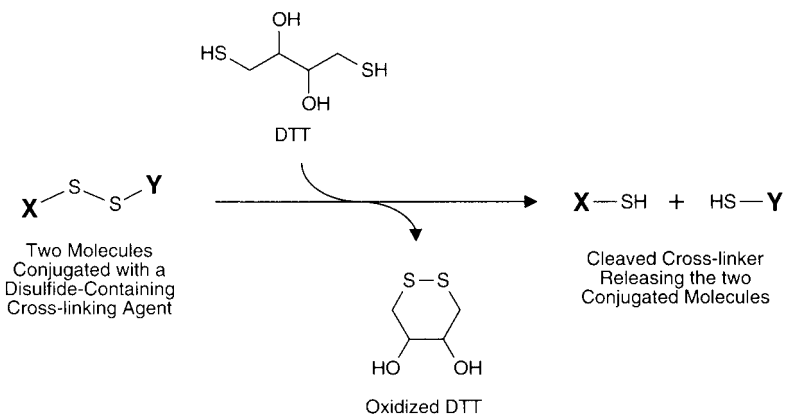


Figure 8.1 Cleavage of disulfide-containing crosslinking compounds can be done using a reducing agent such as DTT. Reduction causes the conjugates to break apart into their original components with each component containing a portion of the crosslinker that terminates in a thiol group.

which will contain a portion of the crosslinker, and on both molecules a free sulfhydryl will be created. Caution should be used with this method of cleavage, however, if other disulfides are present in the conjugated molecules. Some protein disulfides, for instance, also may be affected by the reduction step. Complete cleavage of all disulfides in crosslinked proteins by inclusion of unfolding agents (i.e., guanidine) may yield additional protein fragments of lower molecular weight due to subunit disassociation.

2. Periodate-Cleavable Glycols

Crosslinking agents can be designed to contain adjacent carbon atoms possessing hydroxyl groups. Cross-bridges containing such diols or glycol residues can be constructed from the inclusion of an internal tartaric acid spacer or similar compound in their synthesis (e.g., DST, Chapter 4, Section 1.3). These groups can be easily cleaved by oxidation with sodium periodate (Chapter 1, Section 4.4). Treatment with 15 mM periodate at physiological pH will break the carbon–carbon bond between the glycol portion, oxidizing each hydroxyl to an aldehyde, and cleaving the associated crosslinked molecules (Figure 8.2). Under these conditions, glycosylated portions of glycoproteins or other carbohydrate-containing molecules also will be affected, forming additional aldehyde groups. In some cases, the production of aldehyde residues may cause secondary reactions to occur, especially Schiff base formation with available amine groups. To avoid unexpected crosslinks that form through such intermolecular Schiff base formation, Tris or ethanolamine may be included to tie up the aldehydes as they form.

Sodium periodate also may affect tryptophan residues in some proteins. The oxidation of tryptophan can result in activity losses if the amino acid is an essential component of the active site. For instance, avidin and streptavidin may be severely inactivated by treatment with periodate, since tryptophan is important in forming the biotin-binding pocket. In addition, many other amino acid residues are susceptible to oxidation by periodate (Chapter 1, Section 1.1). Limiting the time of oxidation is important to restricting oxidation to diol groups while not affecting other protein structures.

The use of periodate as a cleavage agent does have advantages, however. Unlike the use of cleavable crosslinkers that contain disulfide bonds which require a reductant to break the conjugate, cleavage of diol-containing crosslinks with periodate typically preserves the indigenous disulfide bonds and tertiary structure of proteins and other molecules. As a result, with most proteins bioactivity usually remains unaffected after mild periodate treatment.

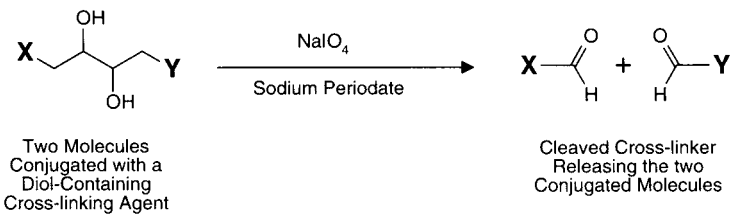


Figure 8.2 Crosslinkers containing a diol group in their cross-bridge design may be cleaved by oxidation with sodium periodate.

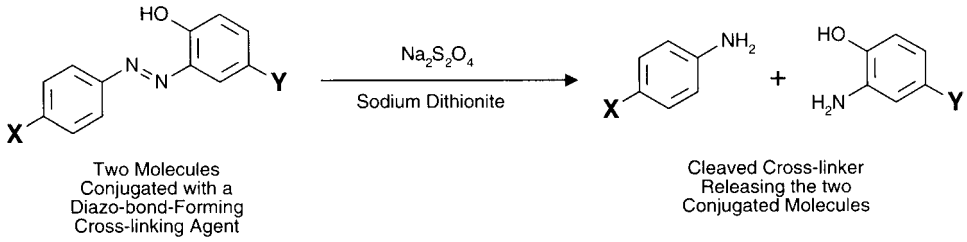


Figure 8.3 Crosslinking agents that form diazo bonds may be cleaved using sodium dithionite.

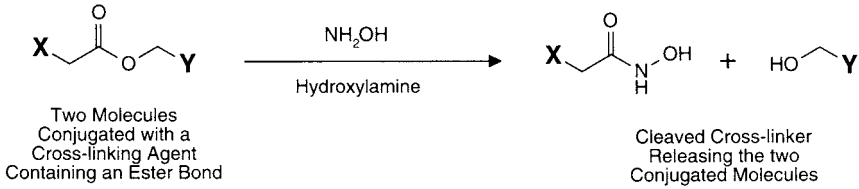


Figure 8.4 Crosslinkers containing an ester group in their cross-bridge are susceptible to cleavage under alkaline conditions using hydroxylamine.

3. Dithionite-Cleavable Bonds

Crosslinking compounds containing diazo bonds within their structures can be specifically cleaved with dithionite (Jaffe *et al.*, 1980). In addition, crosslinks formed by the reaction of a diazonium compound (Chapter 2, Section 6.1) with a tyrosine residue also can be broken using this reagent. Sodium dithionite (also called sodium hydrosulfite) reduces the diazo linkage, breaking the bond between the nitrogens, and leaving a primary amine on both fragments of the crosslinker (Figure 8.3). The reaction usually is carried out in alkaline conditions; a 25-minute incubation with 0.1 M dithionite in 0.2 M sodium borate, pH 9, works well. As the diazo bonds are broken, any color associated with the reagent will disappear.

Dithionite also is capable of cleaving oxime linkages that are formed as the result of the reaction of an aldehyde and aminoxy group (Pojer, 1979). Thus, crosslinks formed between two proteins or other molecules using this reaction strategy can be specifically broken using sodium dithionite.

4. Hydroxylamine Cleavable Esters

Hydroxylamine is a powerful nucleophile which, under alkaline conditions, is effective in breaking ester bonds. Crosslinking agents containing esterified spacer components can be cleaved after undergoing a conjugation reaction by incubation with 0.1 M hydroxylamine, pH 8.5, for 3–6 hours at 37°C (Abdella *et al.*, 1979). The reaction results in the formation of an amide derivative on one fragment of the cleaved crosslinker and a hydroxyl group on the other fragment (Figure 8.4). Thioester bonds also are susceptible to cleavage under these conditions.

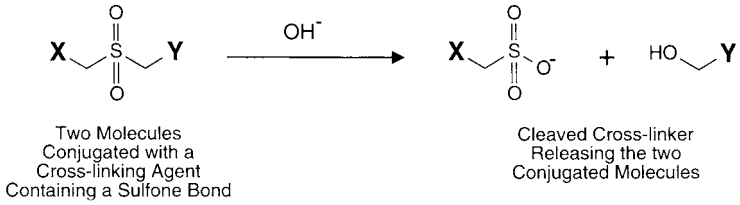


Figure 8.5 Crosslinkers that have an internal sulfone group in their cross-bridge may be cleaved using base.

Thioesters may be broken with the production of an amide and a sulfhydryl group on either side of the crosslinker fragments.

An example of a hydroxylamine-cleavable reagent is EGS (Chapter 4, Section 1.5) which contains two ester bonds made by the esterification of ethylene glycol with succinic acid. Cleavage with hydroxylamine yields two fragments terminating with an amide bond and concomitant release of ethylene glycol. Mass spec evidence of the cleavage products from an EGS crosslink indicates that the cleaved parts can undergo cyclization to form a succinimide group on both fragments (Petrotchenko *et al.*, 2005).

5. Base Labile Sulfones

The presence of a sulfone group in a crosslinking reagent can allow for cleavage of a conjugate through hydrolysis of the linkage under basic conditions. In 0.1 M sodium phosphate, adjusted to pH 11.6 by addition of Tris base, containing 6 M urea, 0.1 percent SDS, and 2 mM DTT, sulfone groups were successfully cleaved after incubation at 37°C for 2 hours (Zarling *et al.*, 1980). In that study, peptide antigens on the surface of lymphocyte receptors were crosslinked with the homobifunctional, amine-reactive reagent BSOCOES (Chapter 4, Section 1.4), purified, and cleaved for analysis. The presence of urea, SDS, and DTT were not absolutely necessary for breaking the sulfone bond, rather they served to disrupt completely protein–peptide structure for complete dissociation of the complex.

In addition to BSOCOES, the amine-reactive, *bis*-fluorobenzene reagent DFDNPS (Chapter 4, Section 4.2) also contains an internal sulfone group that is easily cleaved under basic conditions (Wold, 1961, 1972). Hydrolysis of the sulfone yields two crosslinker fragments, one terminating in a sulfonic acid group and the other containing a hydroxyl group (Figure 8.5).

9

Fluorescent Probes

Labels, tags, and probes are relatively small modifying agents that can be used to label proteins, nucleic acids, and other molecules. These compounds often contain groups that provide sensitive detectability by virtue of some intrinsic chemical or atomic property such as fluorescence, visible chromogenic character, radioactivity, or bioaffinity toward another protein. Most probes can be designed to contain a reactive portion capable of coupling to the functional groups of biomolecules. After modification of a protein via this reactive part, the probe becomes covalently attached, thus permanently tagging it with a unique detectable property. Subsequent interactions that the labeled protein is allowed to undergo can be followed through the tag's visibility.

Labeling molecules by adding a radioactive component was one of the first means of creating highly sensitive detection capabilities. Covalent modification of activated aromatic rings with ^{125}I or ^{131}I easily can be done through tyrosine side chains in proteins or by the use of a phenolic-ring-containing modification agent such as the Bolton–Hunter reagent (Chapter 12, Section 5). Other methods of introducing a radioactive isotope involve the intermediary use of metal-chelating modification reagents such as diethylenetriamine pentaacetic acid (DTPA) (Chapter 10, Section 1). Heavy metal isotopes may be held in coordination complexes on a protein or other molecule and provide extreme sensitivity for *in vivo* diagnostic procedures involving the detection of malignancies. Such complexes coupled to monoclonal antibodies also are being used in the treatment of cancer by their ability to cause cell death in proximity to the bound radiolabel.

Detection of probes or labels usually takes one of three general forms: spectrophotometric, radiosensitive detectors, or indirectly through another labeled substance. Spectral probes can be of two types, chromogenic or fluorescent. Chromogenic labels typically are reserved for non-covalent staining of gross structural features within cells and tissues, as these are present at relatively high concentration. The sensitivity of visible wavelength dyes often is not good enough to provide detectability for low-concentration antigens or low-copy proteins. Even if a protein is covalently modified with a chromogen, the number of associated dye molecules needed to detect it just through its absorbance properties could be prohibitively large to make it viable.

Fluorescent labels, by contrast, can provide tremendous sensitivity due to their property of discrete emission of light upon excitation. Proteins, nucleic acids, and other molecules can be labeled with fluorescent probes to provide highly receptive reagents for numerous *in vitro* assay procedures. For instance, fluorescently tagged antibodies can be used to probe cells and tissues for the presence of particular antigens, and then detected through the use of fluorescence microscopy techniques. Since each probe has its own fluorescence emission character, more

than one labeled molecule—each tagged with a different fluorophore—can be used at the same time to detect two or more target molecules.

A fluorescent molecule has the ability to absorb photons of energy at one wavelength and subsequently emit energy at another wavelength. The absorption process is also called excitation, since the quantum energy levels of some of the compound's electrons increase with photon uptake. The absorption band is not isolated at a discrete photon energy level, but spread out over a range of wavelengths with at least one peak of maximal absorbance within this spectral region. The extinction coefficient (ε, expressed as $M^{-1}\,cm^{-1}$) at the absorbance peak maximum is a unique characteristic of each fluorophore under a given environmental condition.

The excess energy of an excited fluorophore can be lost as heat or through collisions with adjacent molecules or released as photons of light as the electrons return to the lower, ground-state energy level (Figure 9.1). This process of light emission occurs in less than 10^{-4} seconds after

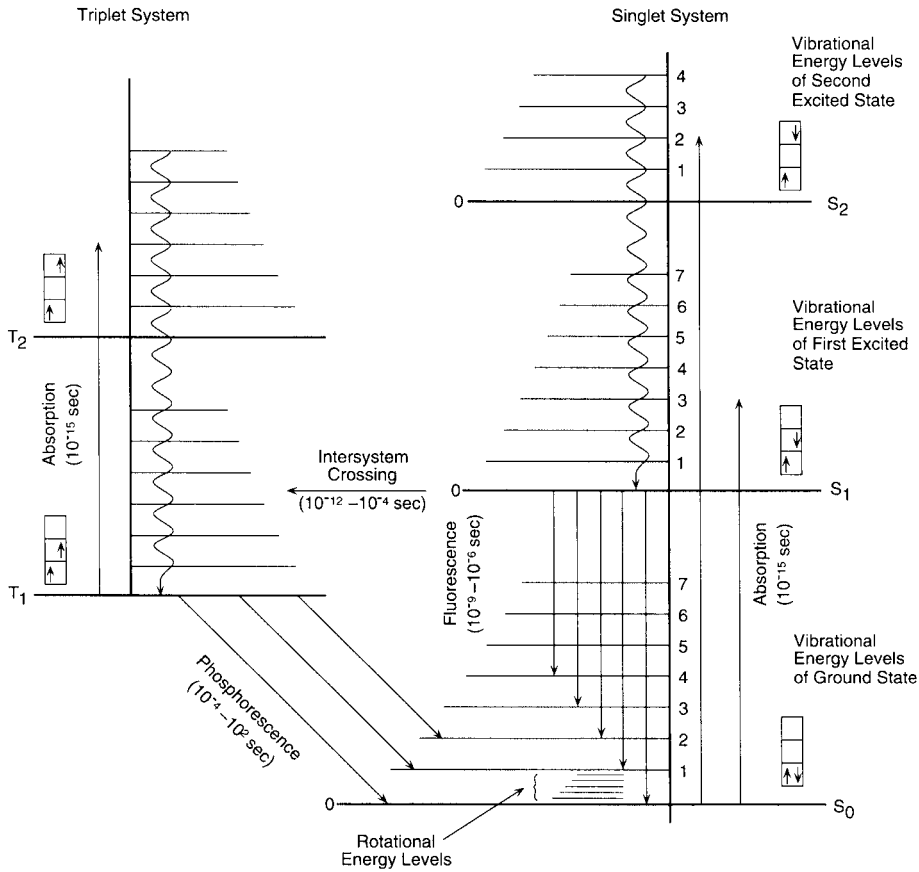


Figure 9.1 Energy diagram showing the transition states involved in the absorption and decay of electromagnetic energy. Energy may be released through heat or internal collisions, transferred to other molecules, or it may be emitted as photons of light. Fluorescence occurs from within the singlet system as light energy is released, returning the electrons to the ground state. Phosphorescence occurs from the triplet system and involves a longer emission process at lower energies than that of fluorescence.

excitation and is known as fluorescence. Fluorescence takes place from the lowest excited singlet state. According to Stoke's Law, the emission wavelength is always longer and thus of lower energy than the wavelength of excitation (Kawamura Jr., 1977). The ratio of total photon emission over the entire range of fluorescence to the total photon absorption is called the quantum yield (QY). QY values range from 0 to 1. The larger the QY value the more efficient the photon emission or luminescence. For a particular fluorophore under fixed environmental conditions, both its extinction coefficient and its QY are fundamental characteristics of its photochemical behavior.

A fluorescent compound suitable for analytical studies should have not only a high QY, but its fluorescence emission spectrum should be separated sufficiently from its excitation spectrum to assure good signal isolation. A fluorophore's Stoke's shift is a measure of the separation of its maximal absorbance wavelength from its emission wavelength maxima (Figure 9.2). The greater the Stoke's shift, the better the signal isolation and therefore less interference from Rayleigh-scattered excitation light.

The majority of reported fluorophores of practical use in labeling biomolecules contain an aromatic ring system as the generator of luminescence. In general, as the conjugated electron system gets larger, the emission wavelength is shifted to the red. Also, the extinction coefficient and QY of larger conjugated systems typically are greater than that of small aromatic compounds.

Aromatic ring constituents can have a pronounced effect on fluorescence. The presence of ring activators or electron donating groups (e.g., *ortho* and *para* directors) generally increases QY, while the presence of electron-withdrawing groups (e.g., *meta* directors) reduces QY. An example of this effect can be found in the photoreactive heterobifunctional crosslinker SAED (Chapter 5, Section 3.9). Before photoactivation, SAED possesses a photoreactive phenyl azide group on its AMCA-derived end. This electron-withdrawing group quenches the fluorescence of the compound so that the AMCA group does not behave as it characteristically does in the underivatized state. Upon photolysis, however, the phenyl azide either gets coupled to a target molecule

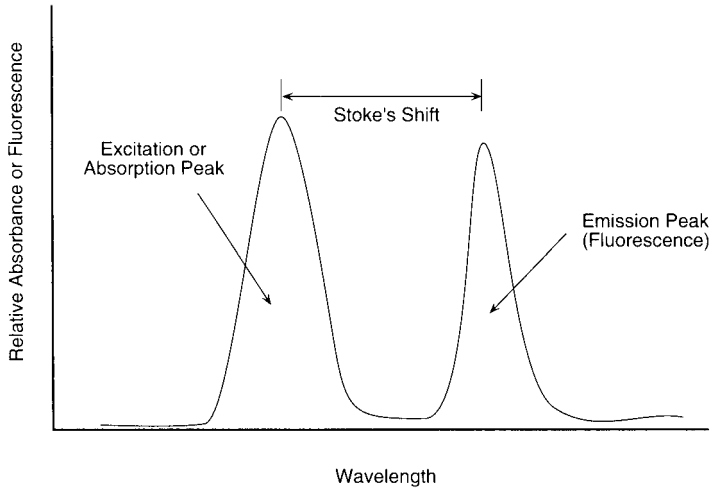


Figure 9.2 Typical spectral scan of a fluorescent compound showing its absorbance peak or wavelengths of most efficient excitation and its emission peak or wavelengths where light emission occurs. The Stoke's shift is the distance in nanometers between the absorbance peak and the emission peak. The larger the Stoke's shift, the less interference that will occur from excitation light when measuring fluorescence emission.

or breaks down to an amine. In either case, the presence of an amine (or its derivative) provides enough ring-activating properties to restore the coumarin group's fluorescent character.

Another potential ring constituent having dramatic affect on fluorescence is the presence of heavy atoms. Aromatic rings possessing heavy atoms diminish QY by enhancing the probability of the excited singlet state going on to triplet transition. Energy decay from a triplet excited state causes phosphorescence instead of fluorescence. The phosphorescent band is located at longer wavelengths (and thus lower energies) relative to the fluorescent spectrum. The energy transition to the triplet state therefore is in direct contention with fluorescence, and has the effect of decreasing overall luminescence. In this regard, the relative fluorescent quenching effect of halogen substitution on aromatic-ring-containing fluorophores is: $I > Br > Cl > F$. In practice, the presence of a chloride atom on a fluorescent aromatic ring may still allow some luminescence to occur; however, the presence of bromide or iodine atoms nearly guarantees complete elimination of fluorescence. Thus, radioiodination of a fluorescent ring structure is not possible without dramatically decreasing (or eliminating) its QY.

The aromatic ring systems of most of the following fluorophores consist of polycyclic structures. To maintain fluorescence in such compounds, it is important that the entire system be coplanar or the rings be in the same dimensional plane. In fact, the differences between some nonfluorescent chromogenic dyes and their corresponding, structurally similar fluorophoresare minor, but the planer nature of the fluorophores gives them their luminescent properties. For instance, Figure 9.3 illustrates the similarity of the dyes phenolphthalein and malachite green to the nearly identical fluorescent molecules fluorescein and rhodamine, respectively. The only difference among

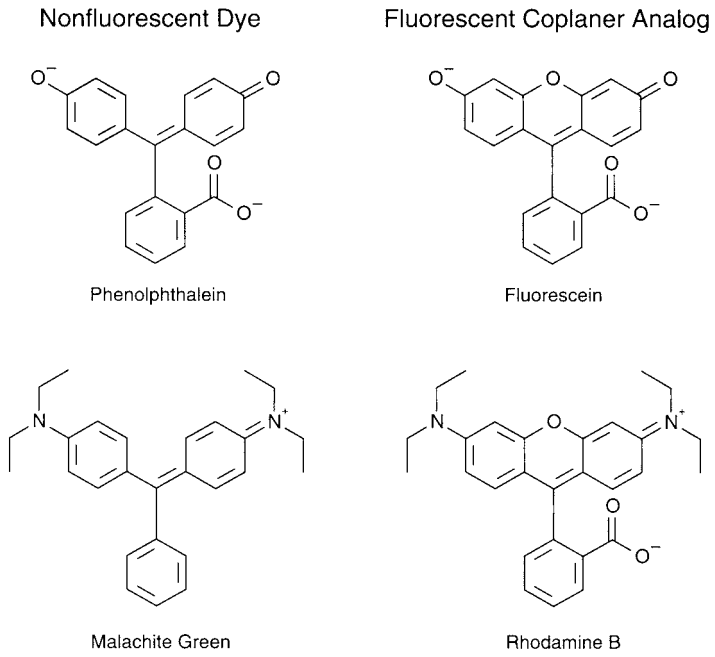


Figure 9.3 Fluorescent character in organic compounds is often determined by the presence of a planar aromatic ring system. The fluorescent compounds differ only in the closure of their central ring system, which produces the required constraints to create a planar triple-ring configuration.

these dyes and fluorophores is the presence of the oxygen bridge between the upper phenyl rings which constricts the molecule to a planer shape, thus conferring luminescent qualities.

Fluorescent compounds are sensitive to changes in their chemical environment. Alterations in media pH, buffer components, solvent polarity, or dissolved oxygen can affect and quench the QY of a fluorescent probe (Bright, 1988). The presence of absorbing components in solution which absorb light at or near the excitation wavelength of the fluorophore will have the effect of decreasing luminescence. In addition, noncovalent interactions of the probe with other components in solution can inhibit rotational freedom and quench fluorescence. In this regard, the binding of an anti-fluorescein antibody to the fluorescent dye completely abolishes its fluorescence.

Over-labeling with a fluorescent probe also causes decreases in QY. Quenching caused by interactions between fluorescent molecules often occurs as the level of probe substitution reaches about 8–10 fluorophores per protein. Not only can the emission intensity decrease severely at high substitution levels, but the degree of nonspecific binding caused by the number of aromatic groups attached to the biomolecule can increase severely. Fluorophore self-quenching at high substitution or concentration levels can be due to energy transfer from excited-state molecules to ground-state dimers (Chen and Knutson, 1988).

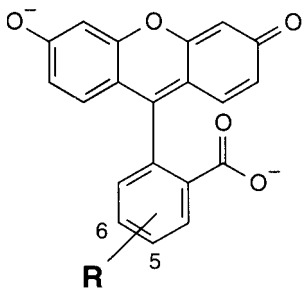
One method of reducing dye–dye interactions is to add negative charge character to the fluorescent molecule. This typically is done by the addition of sulfonate groups on aromatic rings or on alkyl tethers built off the central dye structure. The high negative charge character of the sulfonate groups creates like charge repulsion between dye molecules, thus dramatically reducing their tendency to interact. The presence of three to four sulfonate groups on a probe usually creates a very hydrophilic molecule with low nonspecific binding character in biological solutions and greater QY than the corresponding dye without sulfonates.

The following sections describe some of the most popular fluorescent probes for use in labeling biomolecules. These fluorophores are available from a number of manufacturers in several different forms, with a variety of reactive groups able to couple to specific functional groups on target molecules or with different substituents added to increase QY or hydrophilicity. By far, the most often used fluorescent core structures are derivatives of cyanine, fluorescein, rhodamine, and coumarin—probably in that order. However, new fluorescent molecules are being developed and reported continually. For instance, there is growing interest in the use of lanthanide chelates for time-resolved fluorescence (Section 9, this chapter) or quantum dot nanocrystals (Section 10, this chapter) for highly stable fluorescence in multiplex detection schemes. Careful study of each dye's individual properties can lead to the successful labeling of a biomolecule for any application.

Suggested references on fluorescent spectroscopy and the use of fluorescent probes include Lakowicz (1991, 1999); Bright (1988); Dewey (1991); McGown and Warner (1990); Ploem and Tanke (1987); Darzynkiewicz and Crissman (1990); Haugland (1991); and Waggoner (1990).

1. Fluorescein Derivatives

Fluorescein and its derivatives represent one of the most popular of all fluorescent labeling agents. Its fluorescent character is created by the presence of a multi-ring aromatic structure due to the planer nature of its upper, fused three-ring system (Figure 9.4). In its most elementary



Reactive Fluorescein Derivatives

Figure 9.4 Fluorescein derivatives are produced through modification at the C-5 or C-6 positions on the lower ring.

form, the molecule has a molecular weight of about 332 Da, but most modification reagents based on fluorescein are derivatives of this basic structure prepared through substitutions off the No. 5 or 6 carbons of its lower ring. The derivatives provide reactivity toward particular functional groups in biomolecules, allowing rapid labeling of proteins and nucleic acids.

Fluorescein has an effective excitation wavelength range of about 488–495 nm, closely matching the photon emission for an argon laser (488 nm). Some fluorescein derivatives are sold using the 488 reference in their name to reflect this excitation characteristic (i.e., DyLight 488 from Thermo Fisher and Alexa 488 from Invitrogen). The dye's emission spectrum occurs between 518 and 525 nm, depending on the derivative chosen. Under ideal conditions, its QY can be as high as 0.75, however its fluorescent intensity fades when it is dissolved in buffers, exposed to light, or stored for extended periods (Kawamura Jr., 1977). In environments below pH 7.0, fluorescein's QY is significantly quenched. In addition, when derivatives of fluorescein are conjugated to proteins, the degree of fluorescent quenching can be as high as 50 percent, especially for relatively hydrophobic forms of the dye. Fluorescein derivatives having multiple sulfonates on them help to alleviate the quenching problem when labeling at higher density, but it doesn't completely eliminate it. Even so, the fluorophore usually maintains excellent detectability in assay systems.

The following sections describe the most important fluorescein derivatives having reactive groups commonly used to label biomolecules.

Amine-Reactive Fluorescein Derivatives

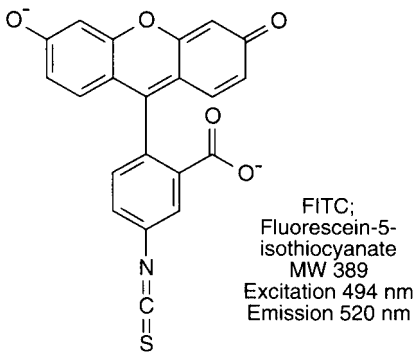
Two general forms of amine-reactive fluorescein probes are available. Both of them react under alkaline conditions with primary amines in proteins and other molecules to form stable, highly fluorescent derivatives.

Fluorescein Isothiocyanate

Fluorescein isothiocyanate (FITC) is one of the most popular fluorescent probes ever created. An isothiocyanate derivative of fluorescein is synthesized by modification of its lower ring at

the 5- or 6-carbon positions. The two resulting isomers are nearly identical in their reactivity and spectral properties, including excitation and emission wavelengths and intensities. Their chemical differences, however, may affect the separation of modified proteins from excess reagent or the analysis of tagged molecules by electrophoresis. For this reason, most manufacturers purify the carbon-5 derivative as the FITC reagent of choice.

Isothiocyanates react with nucleophiles such as amines, sulfhydryls, and the phenolate ion of tyrosine side chains (Podhradsky *et al.*, 1979). The only stable product, however, is with primary amine groups, and so FITC is almost entirely selective for modifying ε- and N-terminal amines in proteins (Jobbagy and Kiraly, 1966). The reaction involves attack of the nucleophile on the central, electrophilic carbon of the isothiocyanate group (Figure 9.5). The resulting electron shift creates a thiourea linkage between FITC and the protein with no leaving group.



FITC can be dissolved in DMF as a concentrated stock solution prior to its addition to an aqueous reaction mixture. This may make aliquoting small quantities of the compound easier. The reagent is water-soluble above pH 6. The isothiocyanate group is reasonably stable in aqueous solution for short periods, but will degrade. FITC also can break down and loose activity upon storage. It is best, therefore, to use fresh reagent for modification purposes. Storage should be done under desiccated conditions, protected from light, and at −20°C.

The fluorescent properties of FITC include an absorbance maximum at about 495 nm and an emission wavelength of 520 nm. Fluorescent quenching of the molecule is possible. Under

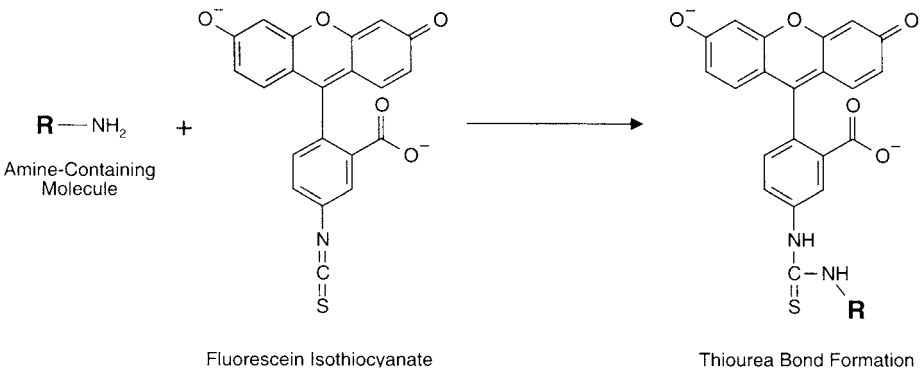


Figure 9.5 FITC reacts with amine-containing compounds to produce an isothiourea linkage.

concentrated conditions, fluorescein-to-fluorescein interactions result in energy transfer and self-quenching, which reduces the luminescence yield. This phenomenon can occur with fluorescein-tagged molecules, as well. If derivatization of a protein is done at too high a level, the resultant QY of the conjugate will be depressed. Typically, modifications of proteins involve adding no more than 8–10 fluorescein molecules per protein molecule, with a 4–5 substitution level considered optimal.

FITC has been used in numerous applications involving fluorescence detection. Antibodies or their fragments can be labeled to detect antigens in cells, tissues sections, blots, or on surfaces (Clausen, 1988). Tagging molecules with FITC also is useful in detecting proteins after electrophoretic separations (Strottmann *et al.*, 1983), for microsequencing analysis of proteins and peptides (Muramoto *et al.*, 1984), in analysis of molecules using capillary zone electrophoresis (Cheng and Dovichi, 1988), and in tracking and detecting molecules involved in various bio-interactions (Burtnick, 1984; Friedman and Ball, 1989).

The level of fluorescein modification in a macromolecule can be determined by measuring its absorbance at or near its characteristic excitation maximum (~498 nm). The number of fluorochrome molecules per molecule of protein is known as the *F/P* ratio. This value should be measured for all derivatives prepared with fluorescent tags. The ratio is important in predicting the behavior of antibodies labeled with FITC (Hebert *et al.*, 1967; Beutner, 1971). Using the known extinction coefficient of FITC in solution at pH 13 ($\varepsilon_{498nm} = 8.1–8.5 \times 10^4$; McKinney *et al.*, 1964; Jobbagy and Jobbagy, 1973) a determination of derivatization level can be made after excess FITC is removed. At pH 7.8, the absorbance of protein-coupled FITC decreases by 8 percent (van Dalen and Haaijman, 1974).

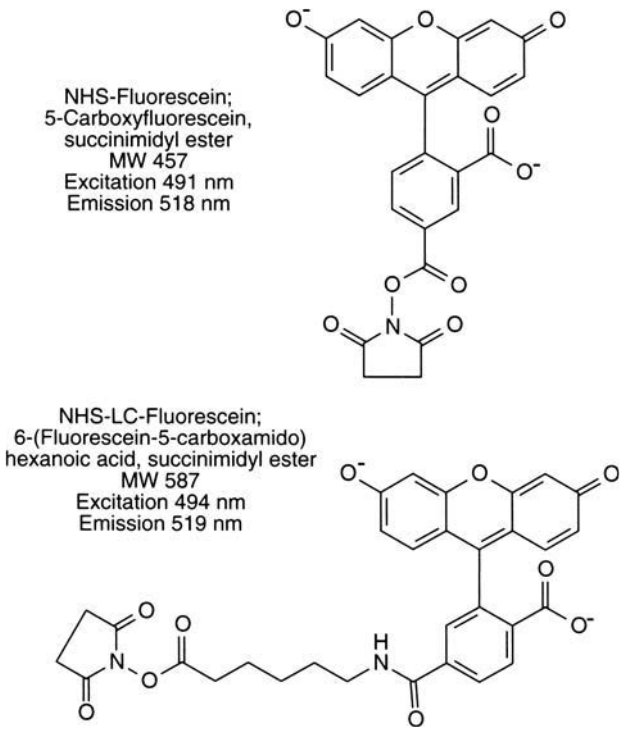
A general protocol for the modification of proteins, particularly immunoglobulins, with FITC is given below. Slight modifications to the amount of reagent added to the reaction may be done to optimize the *F/P* ratio.

Protocol

1. Prepare a protein solution in 0.1 M sodium carbonate, pH 9.0, at a concentration of at least 2 mg/ml.
2. In a darkened lab, dissolve FITC (Thermo Fisher) in dry DMSO at a concentration of 1 mg/ml. Do not use old FITC, as breakdown of the isothiocyanate group over time may decrease coupling efficiency. Protect from light by wrapping in aluminum foil or using amber vials.
3. In a darkened lab, slowly add 50–100 µl of FITC solution to each ml of protein solution (at 2 mg/ml concentration). Gently mix the protein solution as the FITC is added.
4. React for at least 8 hours at 4°C in the dark.
5. The reaction may be quenched by the addition of ammonium chloride to a final concentration of 50 mM. Some protocols also include at this point the addition of 0.1 percent xylene cylanol and 5 percent glycerol as a photon absorber and protein stabilizer, respectively. React for a further 2 hours to stop the reaction by blocking remaining isothiocyanate groups.
6. Purify the derivative by gel filtration using a PBS buffer or another suitable buffer for the particular protein being modified. The use of a desalting resin with low exclusion limits work well. To obtain complete separation, the column size should be 15–20 times the size of the applied sample. Fluorescent molecules often nonspecifically stick to the gel filtration support, so reuse of the column is not recommended.

NHS-Fluorescein and NHS-LC-Fluorescein

N-hydroxysuccinimide (NHS)-fluorescein is another amine-reactive fluorescent probe that contains a carboxy-succinimidyl ester group off the No. 5 or 6 carbons on fluorescein's lower-ring structure (Khanna and Ullman, 1980; Vigers *et al.*, 1988; Brinkley, 1992). The 5- and 6-isomers are virtually identical in their reactivity and fluorescent characteristics. Similar to FITC (above), NHS-fluorescein can be used to label proteins and other macromolecules that contain primary amine groups. This reagent is more stable than FITC, especially in storage. The NHS ester reaction proceeds rapidly at slightly alkaline pH values, resulting in a stable, amide-linked derivative (Chapter 2, Section 1.4 and Chapter 4, Section 1; Figure 9.6).



The fluorescent properties of NHS-fluorescein are similar to FITC. The wavelength of maximal absorbance or excitation for the reagent is 491 nm and its emission maximum is 518 nm, exhibiting a visual color of green (Sheehan and Hrapchak, 1980). Its molar extinction coefficient at 491 nm in a pH 8.0 buffer environment is 66,000 $M^{-1}cm^{-1}$. Other components in solution as well as the pH can change this value.

NHS-fluorescein is insoluble directly in aqueous solution and should be dissolved in organic solvent prior to addition of a small aliquot to a buffered reaction medium. Concentrated stock solutions may be prepared in DMSO or DMF. Such solutions are relatively stable if protected from light. Reaction conditions should be maintained at the optimal reactivity for NHS esters—pH 7–9.

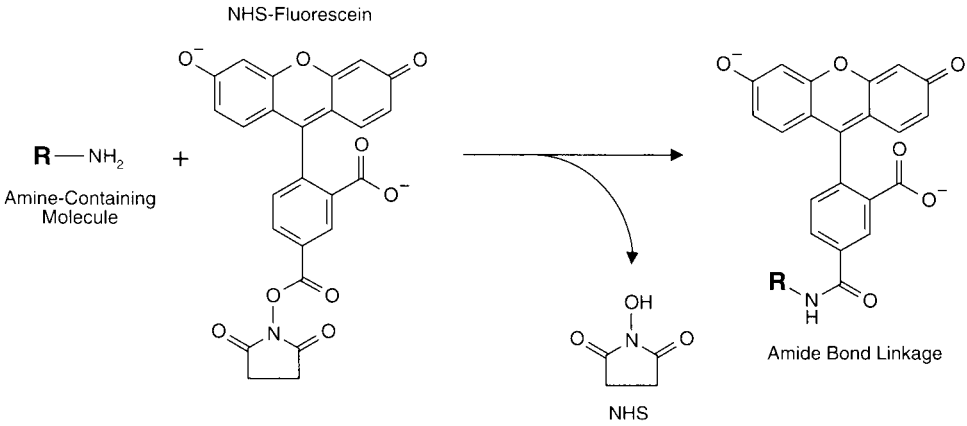


Figure 9.6 NHS-fluorescein reacts with amine-containing compounds via its NHS ester to form amide bonds.

NHS-LC-fluorescein (Invitrogen) is an analog of NHS-fluorescein that contains a 6-aminocaproic acid spacer group, extending the NHS ester group away from the fluorescein portion. The longer length of the coupling arm may decrease steric hindrance around the fluorescent head of the molecule, thus reducing any fluorescence quenching due to its attachment to a macromolecule. All other properties of the long-chain version are virtually identical to that described above for NHS-fluorescein.

Sulfonated versions of NHS-fluorescein dyes are available that contain negative charges to make the molecule more water-soluble and avoid nonspecific interactions with biomolecules (Thermo Fisher and Invitrogen). These 488-type dyes are a better choice than the original fluorescein dyes, because of their advantages when working with proteins and other biological samples, including greater brightness, lower nonspecific binding, and less tendency to quench. The NHS ester reactions of the sulfonated dyes are identical to those of the non-sulfonated types, but the sulfonated ones can be labeled on proteins at higher densities without fluorescence quenching or protein precipitation.

The following procedure is a suggested method for using NHS-fluorescein to label immunoglobulins.

Protocol

1. Dissolve the IgG to be labeled in 50 mM sodium bicarbonate buffer, pH 8.5, at a concentration of 10 mg/ml.
2. Dissolve 0.5 mg of NHS-fluorescein (Thermo Fisher) in 0.5 ml DMSO. Protect from light.
3. In a darkened lab, slowly add 50–100 µl of the NHS-fluorescein solution to the antibody solution, while mixing. Protect from light by wrapping the reaction vessel in aluminum foil.
4. React for 2 hours on ice.
5. Remove unreacted NHS-fluorescein and reaction by-products by gel filtration or dialysis. Continue to protect all labeled protein solutions from light.

A spectrophotometric assessment of the *F/P* ratio should be done after purification of the tagged antibody. The measurement of absorbance at 495 nm (for fluorescein) divided by the absorbance at 280 nm should be between 0.3 and 1.0 to obtain a good fluorescent derivative of acceptable activity and low background. This usually translates into a ratio of about 4–7 fluorescein molecules per protein molecule.

Sulfhydryl-Reactive Fluorescein Derivatives

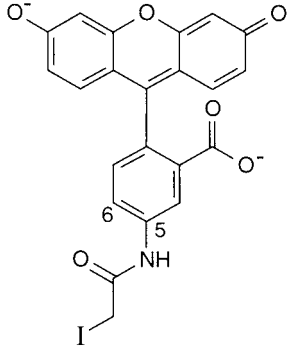
Fluorescein derivatives containing a sulfhydryl-reactive group off the lower-ring structure are available to direct the modification reaction to more limited sites on target molecules. Coupling through sulfhydryls instead of amines can help to avoid active centers in proteins, thus preserving activity in the fluorescent probe complex. Sulfhydryl reaction sites can be naturally available through free cysteine side chains, generated by reduction of disulfides, or created by the use of thiolation reagents (Chapter 1, Section 4.1).

The first two compounds discussed in this section are truly sulfhydryl-reactive, using the common iodoacetyl and maleimide functionalities, respectively. The third derivative, however, is not reactive directly with sulfhydryl groups, but contains a protected sulfhydryl which, after deprotection, can be used to react with other sulfhydryl-reactive crosslinkers.

5-(and 6)-iodoacetamidofluorescein

The iodoacetamido derivatives of fluorescein possess a sulfhydryl-reactive iodoacetyl group (Chapter 1, Section 4.2 and Chapter 2, Section 2.1) at either the 5- or 6-carbon position on their lower ring. The isomers are commercially available in purified form, since some reactivity and specificity differences between the 5- and 6-derivatives toward various sulfhydryl sites in proteins may be observed. Both iodoacetamido derivatives are among the most intense fluorophores available for labeling biomolecules due to high QY.

The iodoacetyl group of both isomers reacts with sulfhydryls under slightly alkaline conditions to yield stable thioether linkages (Figure 9.7). They do not react with unreduced disulfides in cystine residues or with oxidized glutathione (Gorman *et al.*, 1987). The thioether bonds will be hydrolyzed under conditions necessary for complete protein hydrolysis prior to amino acid analysis.



5-(and 6-)-Iodoacetamidofluorescein
MW 515.26
Excitation: 490–495 nm
Emission: 515–520 nm
ε at 492 nm = 80,000–85,000 $M^{-1}cm^{-1}$

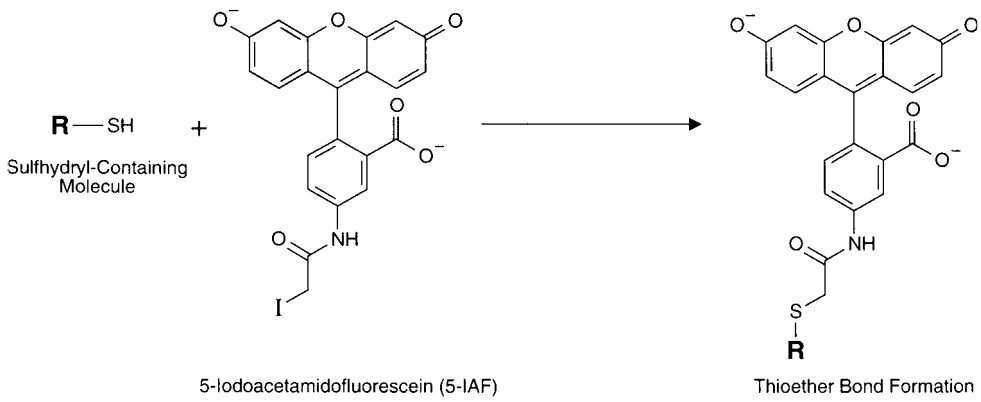


Figure 9.7 5-IAF can be used to modify sulfhydryl-containing molecules, creating stable thioether linkages.

Care must be taken to protect these reagents from light, not only to maintain the fluorescent yield of the fluorescein probe, but also to protect the iodoacetyl group from light-catalyzed breakdown. Iodoacetamidofluorescein is soluble in DMF and also in aqueous solutions maintained above pH 6.0. Concentrated stock solutions may be prepared in DMF prior to addition of a small aliquot to a reaction mixture. Protect solutions from light by wrapping in aluminum foil and working in subdued light.

The spectral properties of these derivatives are similar to native fluorescein. The excitation maximum occurs at about 490–495 nm and its emission peak at 515–520 nm, producing light in the green region of the spectrum. The extinction coefficient of 5-iodoacetamidofluorescein at its wavelength of maximum absorbance, 491 nm, is 82,000 $M^{-1}cm^{-1}$ (pH 9), whereas the extinction coefficient of 6-iodoacetamidofluorescein is 77,000 $M^{-1}cm^{-1}$ at 493 nm (pH 9).

The 5-iodoacetamido derivative of fluorescein (5-IAF) has been used to label numerous proteins and other biomolecules, including actin (Plank and Ware, 1987), myosin (Aguirre *et al.*, 1986), troponin (Greene, 1986), hemoglobin (Hirsch *et al.*, 1986), and sulfhydryl-containing proteins separated by SDS electrophoresis (Gorman, 1984).

The 6-iodoacetamido derivative (6-IAF) has been used to label myosin (Ando, 1984), actin (Konno and Morales, 1985), microtubule-associated proteins (Scherson *et al.*, 1984), and histones (Cocco *et al.*, 1986).

The following protocol for labeling proteins with 5-IAF is adapted from Gorman (1987). It is a bit unusual in that it involves reduction of disulfides with dithiothreitol (DTT) and immediate reaction with 5-IAF in excess without removal of excess reductant. The procedure can be changed to include a gel filtration step after disulfide reduction to remove excess DTT, but in any case, it should be optimized for each protein to be modified. An alternative to the use of DTT to produce sulfhydryls is thiolation with a compound that can generate free thiols upon reaction with a protein (Chapter 1, Section 4.1).

Protocol

1. Dissolve a protein-containing disulfide residues at a concentration of 5–10 mg/ml in 0.1 M ammonium carbonate containing 1 percent SDS and 20 mM DTT. *Note*: The presence of detergent may be eliminated if certain disulfides can be reduced in the protein

without completely denaturing it, such as in the reduction of antibodies in the hinge region. If only partial reduction is done, then the amount of DTT should be reduced to about a 3-fold molar excess over the concentration of antibody present (Sun *et al.*, 2005). For this type of labeling the reaction buffer should be 50 mM sodium borate, pH 8.5.

2. For complete reduction of all disulfides in the presence of a denaturant, react for 16 hours at 0°C and 2 hours at room temperature. For partial reduction of disulfides, the reaction time may be reduced to 2 hours at 37°C, particularly for antibody thiol reduction, if only partial reduction of thiols in the hinge region is done.
3. Add a 5-fold molar excess of 5-IAF (Thermo Fisher) over the amount of DTT present. The fluorescent probe may be solubilized in DMF prior to addition of a small aliquot to the reaction mixture. Do not exceed 10 percent DMF in the final aqueous solution.
4. React for 2 hours at room temperature in the dark.
5. To recover a protein that has been completely reduced and labeled, precipitate the protein by the addition of 9 volumes of methanol at −20°C. Collect the protein pellet by centrifugation at 8,000 g for 15 minutes (4°C). If partial reduction was done followed by labeling with 5-IAF, then purify the labeled protein by gel filtration or dialysis using a molecular weight cutoff of about 5,000 D.

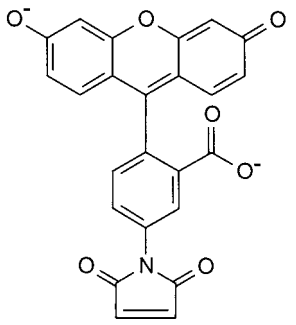
An alternative protocol for labeling sulfhydryl-containing proteins that does not require DTT reduction can be found in a method adapted from Ando (1984). When preparing any fluorescently labeled protein, optimization of the dye-to-protein ratio is important to obtain the best performance in the intended application.

Protocol

1. Prepare a 20 mM 6-IAF (Thermo Fisher) solution by dissolving 10.3 mg/ml of DMF. Prepare fresh and protect from light.
2. Dissolve the protein to be modified at a concentration of 5–10 mg/ml in 20 mM TES, pH 7.0. TES is 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.
3. Slowly add 25–50 µl of the 6-IAF solution to each ml of the protein solution while mixing.
4. React for 2 hours at 4°C in the dark.
5. Remove excess reactant and reaction by-products by gel filtration using a desalting resin or dialysis.

Fluorescein-5-maleimide

Fluorescein-5-maleimide is a fluorescent probe containing a sulfhydryl-reactive maleimide group on its lower-ring structure. Modification of sulfhydryl-containing molecules under physiological pH conditions results in stable thioether bonds (Chapter 2, Section 2.2) (Figure 9.8). The derivative thus possesses fluorescent properties closely characteristic of fluorescein molecules: excitation wavelength = 490 nm; emission wavelength = 515 nm, in the green spectral region. Conjugates prepared by fluorescein-5-maleimide are among the most intensely fluorescent probes available. The reactivity of the maleimide group is similar to that of the iodoacetyl derivative discussed previously.



Fluorescein-5-maleimide
MW 427
Excitation: 490–495 nm
Emission: 515–520 nm
ε at 490 nm = 83,000 $M^{-1}cm^{-1}$

Thus, this reagent can be used to label fluorescently proteins and other biomolecules containing free sulfhydryl residues. If there are no —SH groups available, their creation can be accomplished by reduction of indigenous disulfides or through the use of various thiolation reagents (Chapter 1, Section 4.1).

Fluorescein-5-maleimide is slightly soluble in aqueous solutions above pH 6 (~1 mM concentration). It may be dissolved in DMF at higher concentrations and a small addition of this solution made to an aqueous reaction mixture to initiate labeling. Do not exceed 10 percent DMF in the reaction buffer to avoid protein precipitation. At pH 8, the reagent has an extinction coefficient at 490 nm of about 78,000 M^{-1} cm^{-1}.

Fluorescein-5-maleimide has been used in numerous applications, including labeling the transmembrane glycoprotein H-2K^k on both the N- and C-terminal regions to investigate the structure of the molecule (Cardoza *et al.*, 1984), for the determination of two different conformations of the protein actin (Konno and Morales, 1985), in the study of a bacterial sensory receptors (Falke *et al.*, 1988), in the structural mapping of chloroplast coupling factor (Snyder and Hammes, 1984, 1985), for localization of the stilbenedisulfonate receptor on human

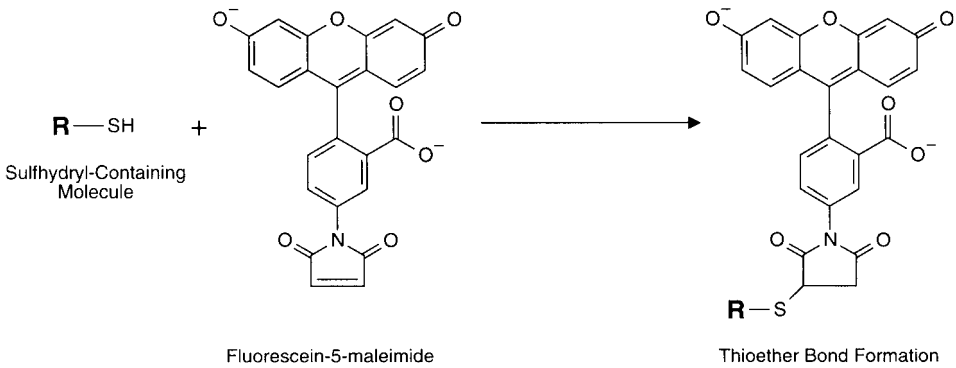


Figure 9.8 Fluorescein-5-maleimide can be used to modify sulfhydryl groups, forming thioether bonds.

erythrocytes (Rao *et al.*, 1979), investigating the calcium-dependent ATPase protein structure of sarcoplasmic reticulum (Bigelow and Inesi, 1991), and to study the movement of tRNA during peptide bond formation on ribosomes (Odom *et al.*, 1990).

Fluorescein-5-maleimide also has been used to study the assembly dynamics of mycobacterium tuberculosis (Chen *et al.*, 2007), to study monomers and dimers of nhaa Na^+/H^+ antiporter of *E. coli* (Rimon *et al.*, 2007), and to investigate the regulation of the protein disulfide proteome by mitochondria (Yang *et al.*, 2007).

Protocol

1. Dissolve a sulfhydryl-containing protein or other macromolecule at a concentration of 1–10 mg/ml in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Other buffers within the range of pH 6.5–7.5 may be used as long as they don't contain extraneous sulfhydryls.
2. Dissolve fluorescein-5-maleimide (Thermo Fisher, Invitrogen) in DMF at a concentration of 10 mM (4.25 mg/ml).Protect from light.
3. In subdued lighting conditions, add 25–50 µl of the fluorescein solution to each ml of protein solution while mixing. Alternatively, determine the exact molar quantity of protein present and add a 25-fold molar excess of fluorescein-5-maleimide solution.
4. React for 2–4 hours at room temperature in the dark. The reaction also may be done at 0–4°C, but allow at least 8 hours for completion.
5. Immediately purify the derivative using gel filtration on a desalting resin. Protect the solutions from light during the chromatography.

SAMSA-Fluorescein

SAMSA-fluorescein, 5-{[2(and 3)-5-(acetylmercapto)-succinoyl]amino}fluorescein, is a fluorescent probe containing a protected sulfhydryl group. In its protected state, the compound is unreactive. The acetyl-protecting group can be removed by treatment with dilute NaOH at pH 10.0 (Figure 9.9). The resulting free sulfhydryl derivative can be used to label thiol-reactive crosslinkers or to couple with sulfhydryl residues on proteins and other molecules. After activating

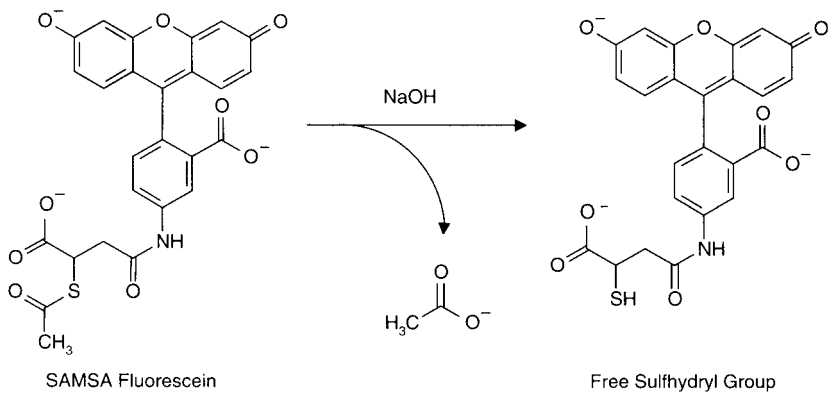
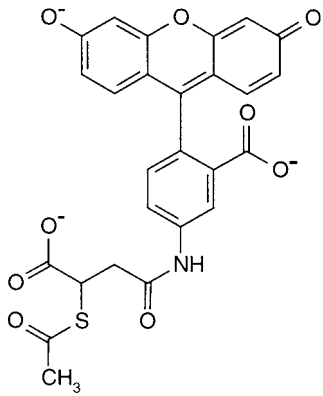


Figure 9.9 SAMSA-fluorescein contains a protect thiol that can be deblocked by treatment with hydroxylamine. The reagent then can be used to modify molecules containing sulfhydryl-reactive groups.

proteins with crosslinkers containing terminal maleimide, pyridyl disulfide, or iodoacetyl groups, SAMSA-fluorescein can be used to assess the level of modification. For instance, a maleimide-activated protein that has been derivatized with succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) could be reacted with this reagent to yield a fluorescein derivative that can be assayed spectrofluorometrically for its level of fluorescence. Using the molar extinction coefficient for SAMSA-fluorescein (ε at 495 nm = 80,000 $M^{-1}cm^{-1}$), the molar level of incorporation of the label can be calculated. This determination directly correlates to the original level of maleimide groups present on the protein.



SAMSA Fluorescein
MW 522
Excitation = 495 nm
Emission = 518 nm

SAMSA-fluorescein is an orange solid compound. Dissolved in buffer at pH 9.0, its maximal wavelength of absorption or excitation is at 495 nm, while its emission wavelength maximum is 520 nm. The reagent and all solutions and derivatives made from it are light sensitive and should be stored in the dark. SAMSA-fluorescein is soluble in aqueous solutions above pH 6.0, but it can be dissolved in DMF to prepare a concentrated stock solution prior to adding a small amount to a buffered reaction mixture.

Protocol

1. To deprotect the acetylated sulfhydryl, dissolve the desired amount of SAMSA-fluorescein (Invitrogen) in 100 mM NaOH, pH 10.0, at a concentration of 10 mg/ml.
2. React for 15 minutes at room temperature.
3. Lower the pH to 7–8 by the addition of solid sodium phosphate.
4. Add the required amount of deprotected SH-fluorescein to a protein or other macromolecule that had been modified to contain a sulfhydryl-reactive group. Use a 5- to 10-fold molar excess of SH-fluorescein to the expected amount of sulfhydryl-reactivity present.
5. React for 2 hours at room temperature, protected from light.
6. Remove excess fluorescent probe by gel filtration using a desalting resin.
7. Measure the absorbance of the derivative at 495 nm. Determine the level of fluorophore incorporation by using its molar extinction coefficient.

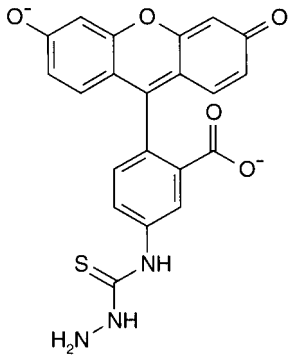
Aldehyde/Ketone and Cytosine-Reactive Fluorescein Derivatives

Hydrazide groups directly react with aldehyde and ketone groups to form relatively stable hydrazone linkages (Chapter 2, Section 5.1). Two fluorescein derivatives are commonly available that contain hydrazide groups off their No. 5 carbons on the lower-ring structure. Both may be used to label fluorescently aldehyde- or ketone-containing molecules. Although most biomolecules don't contain aldehyde or ketone groups in their native state, carbohydrates, glycoproteins, RNA, and other molecules containing sugar residues can be oxidized with sodium periodate to produce reactive formyl groups. The use of modification reagents which generate aldehydes upon coupling to a molecule also can be used to produce a hydrazide-reactive site (Chapter 1, Section 4.4).

DNA and RNA may be modified with hydrazide-reactive probes by reacting their cytosine residues with bisulfite to form reactive sulfone intermediates. These derivatives undergo transamination to couple hydrazide- or amine-containing probes (Draper and Gold, 1980) (Chapter 27, Section 2.1).

Fluorescein-5-thiosemicarbazide

Fluorescein-5-thiosemicarbazide is a hydrazide derivative of fluorescein that can spontaneously react with aldehyde- or ketone-containing molecules to form a covalent, hydrazone linkage (Figure 9.10) (Invitrogen). It also can be used to label cytosine residues in DNA or RNA by use of the bisulfite activation procedure (Chapter 27, Section 2.1). The resulting fluorescent derivative exhibits an excitation maximum at a wavelength of 492 nm and a maximal emission wavelength of 519 nm when dissolved in buffer at pH 8.6. In the same buffered environment, the compound has an extinction coefficient of approximately 78,000 $M^{-1}cm^{-1}$ at 492 nm.



Fluorescein-5-thiosemicarbazide
MW 421
Excitation: 492 nm
Emission: 516 nm
ε at 492 nm = 85,000 $M^{-1}cm^{-1}$

Fluorescein-5-thiosemicarbazide is soluble in DMF or in buffered aqueous solutions at pH values above 7.0. The reagent may be dissolved in DMF as a concentrated stock solution before adding a small aliquot to an aqueous reaction medium. The compound itself and all solutions made with it should be protected from light to avoid decomposition of its fluorescent properties.

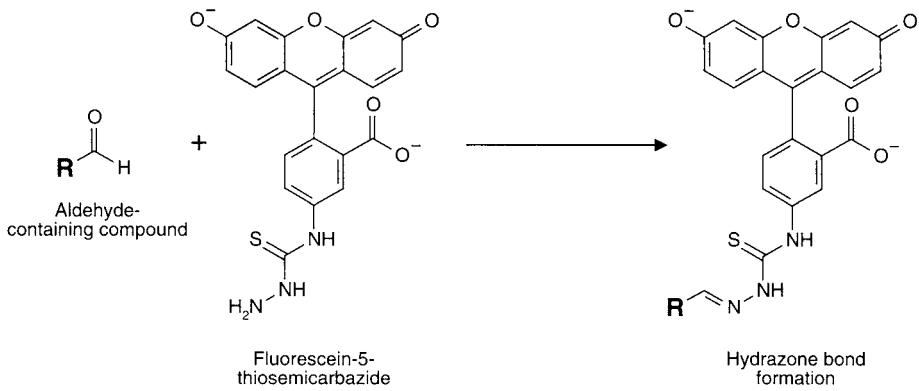


Figure 9.10 Fluorescein-5-thiosemicarbazide reacts with aldehyde groups to produce hydrazone linkages.

This hydrazide derivative of fluorescein has been used in a number of applications, including site-directed labeling of antibodies through their carbohydrate chains (Duijndam *et al.*, 1988), labeling thrombin and anti-thrombin (Atha *et al.*, 1964), the Na^+/K^+-ATPase glycoprotein (Lee and Fortes, 1985), periodate-oxidized RNA (Odom *et al.*, 1980, 1984; Ferguson and Yang, 1986; Friedrich *et al.*, 1988), for the determination of carbonyl groups in proteins and to detect oxidized glycoproteins in gels (Ahn *et al.*, 1987). It also has been used to investigate the molecular basis of RNA recognition (Pagano *et al.*, 2007), in a non-invasive visualization method for assessment of carbonylated protein (Fujita *et al.*, 2007), and for the detection of protein carbonyls in aging liver tissue (Chaudhuri *et al.*, 2007).

The following protocols are generalized for the labeling of cell-surface glycoproteins or glycoproteins in solution. Some optimization may be necessary to achieve the best level of fluorescent modification for each particular application.

Protocol for Labeling Cell Surfaces

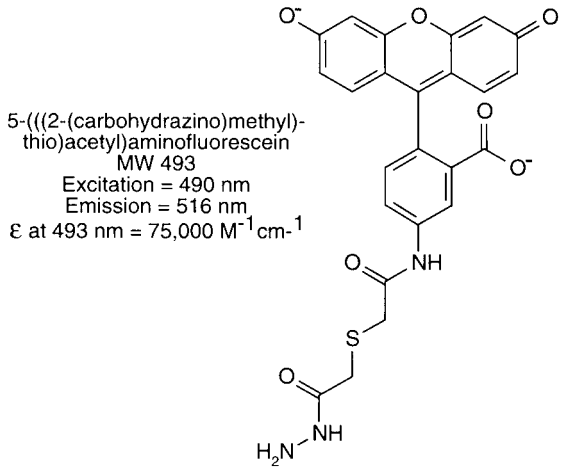
1. Add 10^6–10^8 cells/ml in a PBS solution (10 mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 1 mM sodium periodate and incubate on ice for 30 minutes in the dark. This level of periodate addition will target the oxidation only to sialic acid residues (Chapter 1, Section 4.4). If additional sites of glycosylation also are to be labeled, increase the periodate concentration to 10 mM and do the reaction at room temperature in the dark.
2. Centrifuge and wash cells several times with PBS. Some protocols include a quench step wherein excess sodium periodate is eliminated by addition of glycerol. This can be accomplished by adding 3 volumes of 0.1 M glycerol in PBS prior to centrifugation.
3. Resuspend cells in PBS containing 0.5 mg/ml fluorescein-5-thiosemicarbazide.
4. Incubate 30 minutes in the dark at room temperature.
5. To reduce the hydrazone bonds to more stable linkages, cool the cell suspension to 0°C and add an equal volume of 30 mM sodium cyanoborohydride in PBS. Incubate for 40 minutes. *Note*: If the presence of a reducing agent is detrimental to protein activity, eliminate the reduction step. In most cases, the hydrazone linkage is stable enough for fluorescent labeling experiments.
6. Centrifuge and wash cells extensively with PBS.

Protocol for Labeling Glycoproteins in Solution

1. Dissolve the glycoprotein(s) to be labeled in ice-cold 1 mM sodium periodate, 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4, for the exclusive oxidation of sialic acid residues. For general carbohydrate oxidation, increase the periodate concentration to 10 mM in PBS at room temperature.
2. React for 30 minutes on ice (for sialic acids) or at room temperature (for other polysaccharide residues).
3. Remove excess reactant by gel filtration using a desalting resin with PBS, pH 7.4. Some protocols use a quenching agent to remove excess periodate prior to gel filtration. This can be done by adding glycerol to a final concentration of 0.1 M.
4. To the purified, oxidized glycoprotein(s), add fluorescein-5-thiosemicarbazide to a final concentration of 0.5 mg/ml.
5. React for 30 minutes at room temperature in the dark.
6. To reduce the hydrazone bonds to more stable linkages, cool the solution to 0°C and add an equal volume of 30 mM sodium cyanoborohydride in PBS. Incubate for 40 minutes. *Note*: If the presence of a reducing agent is detrimental to protein activity, eliminate the reducing step. In most cases, the hydrazone linkage is stable enough for fluorescent labeling experiments.
7. Purify the fluorescently labeled glycoprotein(s) by gel filtration using a desalting resin.

5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein

Another hydrazine derivative of fluorescein, 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein, contains a longer spacer arm off its No. 5 carbon atom of its lower ring than fluorescein-5-thiosemicarbazide, described previously (Invitrogen). The reagent can be used to react spontaneously with aldehyde- or ketone-containing molecules forming a hydrazone linkage (Figure 9.11). It also can be used to label cytosine residues in DNA or RNA by use of the bisulfite activation procedure (Chapter 27, Section 2.1). The resulting fluorescent derivative exhibits a maximal excitation at 490 nm and a maximal luminescence emission peak at 516 nm when dissolved in buffer at pH 8.0. In the same buffered environment, the compound has an extinction coefficient of approximately 75,000 $M^{-1}cm^{-1}$ at 490 nm.



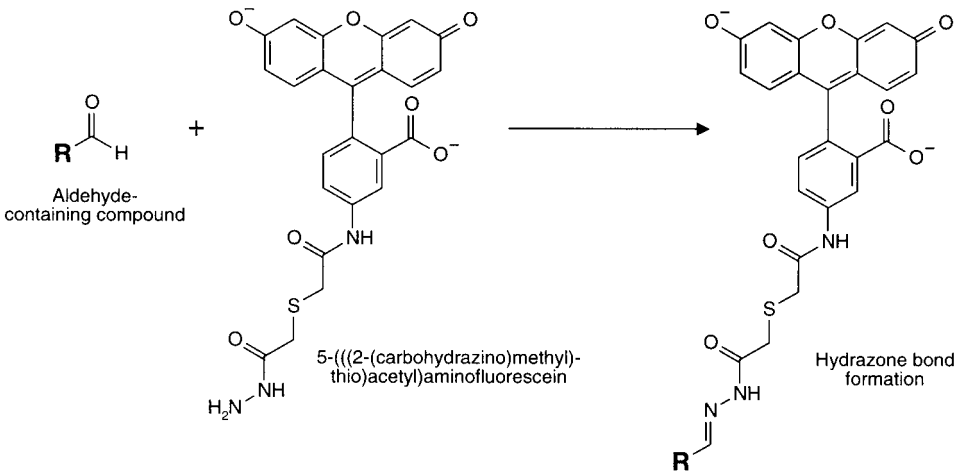


Figure 9.11 This carbohydrazide-containing fluorescein derivative can be used to modify aldehyde-containing molecules. Glycoconjugates may be labeled with this reagent after treatment with sodium periodate to produce aldehydes.

The fluorescent probe, 5-(((2-(carbohydrazino)methyl)thio)acetyl)-aminofluorescein, is soluble in DMF or in buffered aqueous solutions at pH values above 7.0. The reagent may be dissolved in DMF as a concentrated stock solution before adding a small aliquot to an aqueous reaction medium. The compound itself and all solutions made with it should be protected from light to avoid decomposition of its fluorescent properties.

The methods for using this reagent in labeling glycoproteins on cell surfaces or in solution are similar to those described for fluorescein-5-thiosemicarbazide, above.

2. Rhodamine Derivatives

Rhodamine and its derivatives are popular fluorescent probes for labeling all types of biomolecules. Their fluorescent character is created by the presence of a planer, multi-ring aromatic structure similar to fluorescein, but with nitrogen atoms replacing the oxygens on the outer rings (Figure 9.12). Fluorescent modification reagents based on rhodamine are derivatives of this basic structure. Activated rhodamine probes have reactive groups prepared through substitutions off the No. 5 or 6 carbons of its lower ring. These derivatives provide reactivity toward particular functional groups in biomolecules, allowing rapid labeling of proteins and nucleic acids. Other alterations to the basic rhodamine structure modulate its fluorescent character, creating more intense or stable fluorophores, or changing its wavelength of excitation and emission toward the red region. Many such derivatives are now commercially available.

The tetramethylrhodamine derivative, for instance, has two methyl groups attached to each nitrogen on its outer rings. Activated forms of tetramethylrhodamine are among the most common derivatives of rhodamine used for fluorescent labeling. Another useful derivative is rhodamine B, which contains two ethyl groups on each nitrogen as well as a carboxylate group at the No. 3 position on its lower ring. Rhodamine 6G adds two methyl groups on the outer rings as well as an ethyl ester group off rhodamine B's carboxylate. Rhodamine 110 contains no

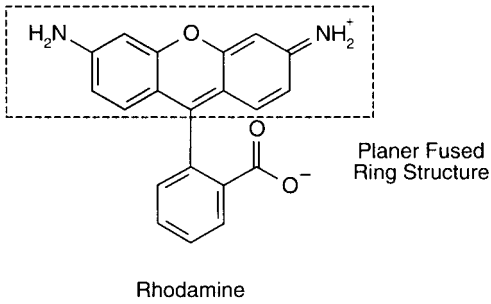


Figure 9.12 The basic structure of rhodamine derivatives.

substituents on the upper nitrogens and only the carboxylate on the lower ring. Sulforhodamine B possesses rhodamine B's two ethyl groups on each nitrogen of the upper rings, but has two sulfonates at the No. 3 and 5 positions of its lower ring. This derivative is often called Lissamine™ rhodamine B—Lissamine being a trademark of Imperial Chemical Industries. Another popular derivative of rhodamine, sulforhodamine 101, goes by the name of Texas Red™ (a trademark of Invitrogen). This derivative has intense luminescent properties that take it the farthest into the red region of the spectrum. The basic structures of these rhodamine derivatives are shown in Figure 9.13. The corresponding commercially available rhodamine fluorophores usually contain additional reactive groups, on the No. 5 or 6 carbons of the lower ring, to permit coupling to target molecules (Invitrogen, Thermo Fisher).

Rhodamine derivatives have effective excitation wavelengths within the visible light spectrum from the low- to high-500 nm range, depending on the particular derivative. Their associated emission wavelengths occur from the mid- to high-500 nm range—with Texas Red derivatives typically emitting at over 600 nm–within the orange-to-red visible spectrum. The QY of rhodamine derivatives is generally less than that of fluorescein–only about 25 percent. However, its fluorescent intensity fades more slowly than fluorescein when it is dissolved in buffers, exposed to light, or stored for extended periods. In addition, its orange-to-red luminescence is in stark contrast to the green of fluorescein, thus these two types of probes form an ideal pair for use in double-staining techniques, especially in fluorescent microscopy.

The following sections describe the most important rhodamine derivatives commonly used to label biomolecules.

Amine-Reactive Rhodamine Derivatives

Four forms of amine-reactive rhodamine probes are commonly available. Two of them are based on the tetramethyl derivatives of the fundamental rhodamine structure, one is based on the sulforhodamine B or Lissamine derivative, and the last is the sulforhodamine 101 or Texas Red-type of derivative. All of them react under alkaline conditions with primary amines in proteins and other molecules to form stable, highly fluorescent complexes.

Tetramethylrhodamine-5-(and 6)-isothiocyanate

Tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC) is one of the most popular fluorescent probes available. The isothiocyanate derivative of tetramethylrhodamine is synthesized by

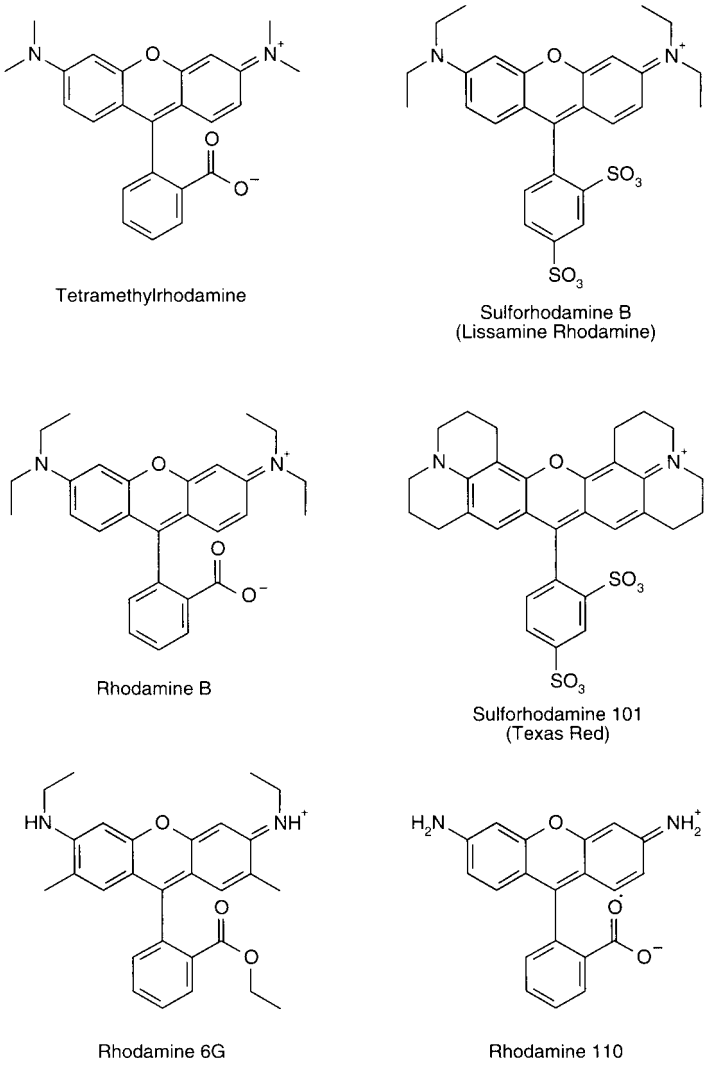


Figure 9.13 The primary rhodamine derivatives useful for fluorescent labeling.

modification of its lower ring at the 5- or 6-carbon positions. The two resulting isomers are almost identical in their reactivity but slightly different in their spectral properties, including excitation and emission wavelengths and intensities. The chemical differences in the isomers, however, may affect the separation of modified proteins from excess probe or the analysis of tagged molecules by electrophoresis. For this reason, most manufacturers offer the mixed isomers as well as the purified 5- or 6-isothiocyanate derivatives individually.

Isothiocyanates react with nucleophiles such as amines, sulfhydryls, and the phenolate ion of tyrosine side chains (Podhradsky *et al.*, 1979). The only stable product, however, is with primary amine groups, and so TRITC is almost entirely selective for modifying ε- and N-terminal amines in proteins. The reaction involves attack of the nucleophile on the central, electrophilic

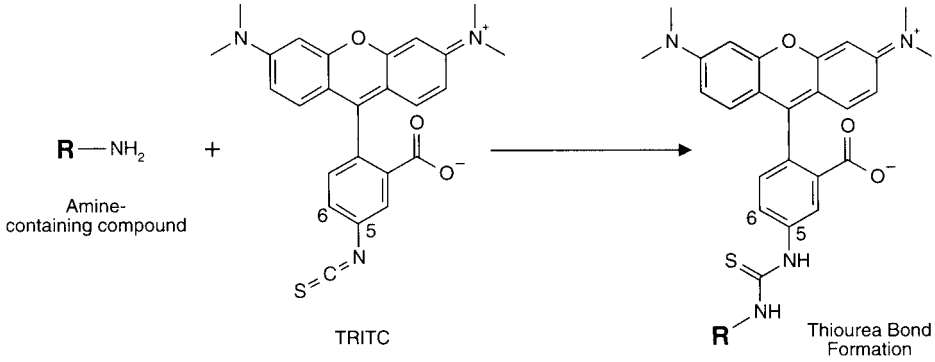
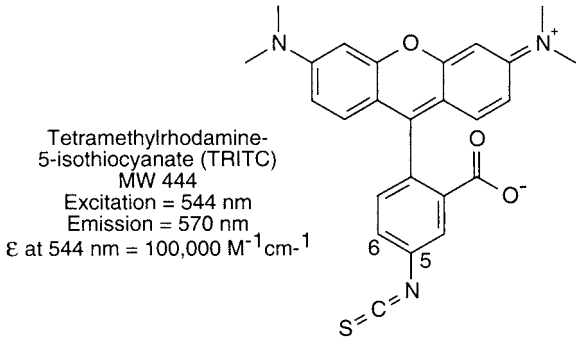


Figure 9.14 TRITC reacts with amine-containing molecules to create an isothiourea linkage.

carbon of the isothiocyanate group (Figure 9.14). The resulting electron shift creates a thiourea linkage between TRITC and the protein with no leaving group.



TRITC is relatively insoluble in water, but it can be dissolved in DMF or DMSO as a concentrated stock solution prior to its addition to an aqueous reaction mixture. The isothiocyanate group is reasonably stable in aqueous solution for short periods, but will degrade by hydrolysis. TRITC also is more stable to photobleaching than FITC (Section 1, this chapter), and its absorption and emission spectra are less sensitive to environmental conditions, such as pH. It is best, however, to use only fresh reagent for modification purposes. Storage should be done under desiccated conditions, protected from light, and at −20°C.

The fluorescent properties of TRITC (mixed isomers) include an absorbance maximum at about 544 nm and an emission wavelength of 570 nm. Fluorescent quenching of the molecule is possible. Under concentrated conditions, rhodamine-to-rhodamine interactions result in self-quenching which reduces its luminescence yield. This phenomenon can occur with TRITC-tagged molecules, as well. If derivatization of a protein is done at too high a level, the resultant QY of the conjugate will be depressed from expected values. Typically, modifications of proteins involve adding no more than 8–10 rhodamine molecules per molecule of protein, with a 4–5 substitution level considered optimal.

TRITC has been used in numerous applications involving fluorescence detection, including double-staining techniques with fluorescein-labeled probes (Mossberg and Ericsson, 1990), the synthesis of fluorescently labeled DNA probes (Smith *et al.*, 1985), as a label in homogeneous

immunoassay systems (Nithipatikom and McGown, 1987), to investigate specific interactions of proteins with cell surfaces (Hochman *et al.*, 1988), and as an important fluorescent tag of antibodies in immunohistochemical staining techniques (Davidson and Hilchenbach, 1990). The fluorescent dye also has been used to investigate dynein-dependent transport of virus proteins (Ramanathan *et al.*, 2007), the trafficking of the prion protein (Campana *et al.*, 2007), and the distribution of FAT1 isoforms in migrating cells (Braun *et al.*, 2007). Many thousands of additional references cite the use of TRITC in labeling molecules in fluorescence detection applications.

The level of TRITC modification in a macromolecule can be determined by measuring its absorbance at or near its characteristic absorption maximum (~575 nm). The number of fluorochrome molecules per molecule of protein is known as the *F/P* ratio. This value should be measured for all derivatives prepared with fluorescent tags. The ratio is especially important in predicting the behavior of antibodies labeled with TRITC. For a TRITC-labeled protein, the ratio of its absorbance at 575–280 nm should be between 0.3 and 0.7.

A general protocol for the modification of proteins, particularly immunoglobulins, with TRITC is given below. Modifications to the amount of reagent added to the reaction may be done to optimize the *F/P* ratio.

Protocol

1. Prepare a protein solution in 0.1 M sodium carbonate, pH 9.0, at a concentration of at least 2 mg/ml.
2. In a darkened lab, dissolve TRITC (Thermo Fisher) in dry DMSO at a concentration of 1 mg/ml. Do not use old TRITC, as breakdown of the isothiocyanate group over time may decrease coupling efficiency. Protect from light by wrapping in aluminum foil or using amber vials.
3. In a darkened lab, slowly add 50 µl of TRITC solution to each ml of protein solution. Gently mix the protein solution as the TRITC is added.
4. React for at least 8 hours at 4°C in the dark or 2–4 hours at room temperature.
5. The reaction may be quenched by the addition of ammonium chloride to a final concentration of 50 mM. Some protocols also include at this point the addition of 0.1 percent xylene cylanol and 5 percent glycerol as a photon absorber and protein stabilizer, respectively. React for a further 2 hours to stop the reaction by blocking remaining isothiocyanate groups.
6. Purify the derivative by gel filtration using a PBS buffer or another suitable buffer for the particular protein being modified. The use of a desalting resin (e.g., Excellulose, Thermo Fisher) or similar matrices with low exclusion limits work well. To obtain complete separation, the column size should be 15–20 times the size of the applied sample. Fluorescent molecules often nonspecifically stick to gel filtration supports, so reuse of the column is not recommended.

NHS-Rhodamine

NHS-rhodamine is an amine-reactive fluorescent probe that contains a carboxy-succinimidyl ester group off the No. 5 or 6 carbons on rhodamine's lower-ring structure (Kellogg *et al.*, 1988). The 5- and 6-isomers are virtually identical in their reactivity and fluorescent characteristics. Similar to TRITC (described previously), NHS-rhodamine can be used to label proteins and other macromolecules that contain primary amine groups. The isomeric forms of the fluorescent probe are available in mixed and purified forms (Invitrogen, Thermo Fisher). The pure forms are

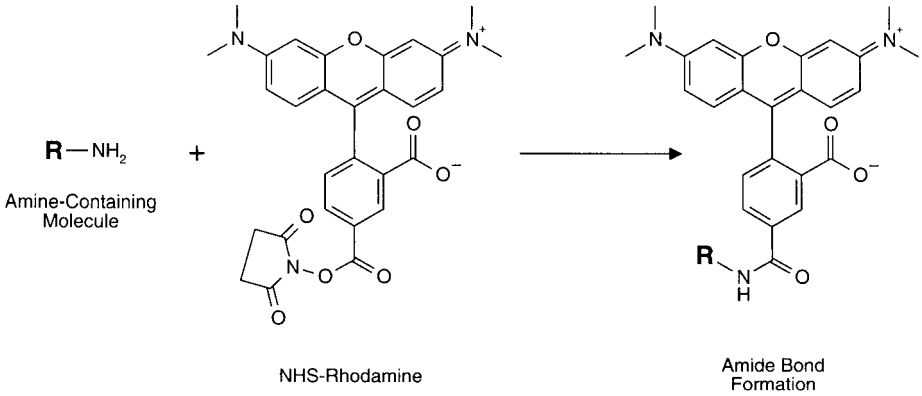
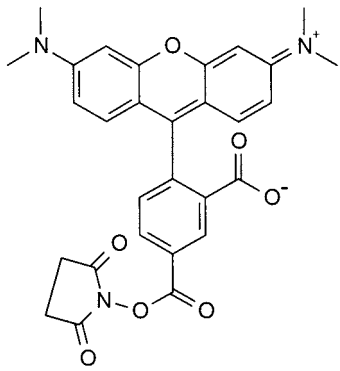


Figure 9.15 NHS-rhodamine can be used to label amine-containing molecules via its NHS ester group.

particularly important for labeling nucleic acid probes that will be separated by electrophoresis (Chehab and Kan, 1989). The NHS ester labeling reaction proceeds rapidly at slightly alkaline pH values, resulting in a stable, amide-linked derivative (Chapter 2, Section 1.4; Figure 9.15).



NHS-Rhodamine;
5-Carboxytetramethyl-
rhodamine, succinimidyl ester
MW 528
Excitation = 546 nm
Emission = 579 nm
ε at 546 nm = 100,000 $M^{-1}cm^{-1}$

The fluorescent properties of NHS-rhodamine are similar to TRITC. The wavelength of maximal absorbance or excitation for the reagent is 544 nm and its emission maximum is 576 nm, exhibiting a visual color of orange-red. Its molar extinction coefficient at 546 nm in a methanol environment is 63,000 $M^{-1}cm^{-1}$. Other components in solution as well as the pH (in aqueous buffers) can change this value.

NHS-rhodamine is insoluble directly in aqueous solution and should be dissolved in organic solvent prior to addition of a small aliquot to a buffered reaction medium. Concentrated stock solutions may be prepared in DMSO or DMF. Such solutions are relatively stable for short

periods if protected from light, but should be prepared fresh. Reaction conditions should be maintained at the optimal reactivity for NHS esters, which is pH 7–9.

NHS-rhodamine has been used in numerous applications, including the detection of specific DNA sequences (Chehab and Kan, 1989), studying the behavior of microtubules and actin filaments in living *Drosophila* embryos (Kellogg *et al.*, 1988), investigation into the light-initiated breakup of microtubules (Vigers *et al.*, 1988), studying ω-conotoxin-sensitive channels in neurons (Jones *et al.*, 1989), investigating growth cones during axon elongation (Tanaka and Kirschner, 1991), and in studying the pathways of mitotic spindle assembly *in vitro* (Sawin and Mitchison, 1991). NHS-rhodamine also has been used to perform fluorescent studies of membrane protein complexes (Wittig *et al.*, 2007), to investigate the cross-bridge between microtubules (Li *et al.*, 2007), and study a multi-enzyme network in digestion (Delcroix *et al.*, 2006).

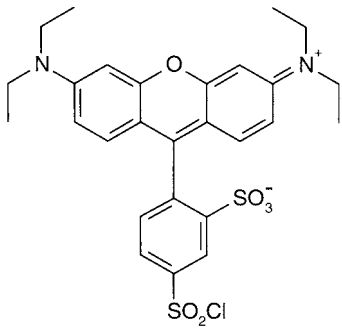
The following generalized protocol relates to the labeling of IgG with NHS-rhodamine. Optimization of the level of rhodamine incorporation may have to be done with other proteins or other macromolecules.

Protocol

1. Dissolve an immunoglobulin to be labeled in ice-cold, 50 mM sodium bicarbonate, pH 8.5, at a concentration of 10 mg/ml.
2. Dissolve NHS-rhodamine at a concentration of 1 mg/ml in DMSO. Protect from light.
3. In a darkened lab, slowly add 50–100 µl of the NHS-rhodamine solution to each ml of the antibody solution with mixing. Wrap the vessel with aluminum foil to protect from light.
4. Place the sample on ice and react for 2 hours.
5. Remove unreacted NHS-rhodamine and reaction by-products by gel filtration or dialysis.

Lissamine Rhodamine B Sulfonyl Chloride

The Lissamine form of rhodamine B consists of diethyl modifications on the two nitrogens of the upper rings of the basic rhodamine molecule as well as two sulfonate groups added at the



Lissamine Rhodamine B
Sulfonyl Chloride
MW 577
Excitation = 556 nm
Emission = 576 nm
ε at 556 nm = 100,000 $M^{-1}cm^{-1}$

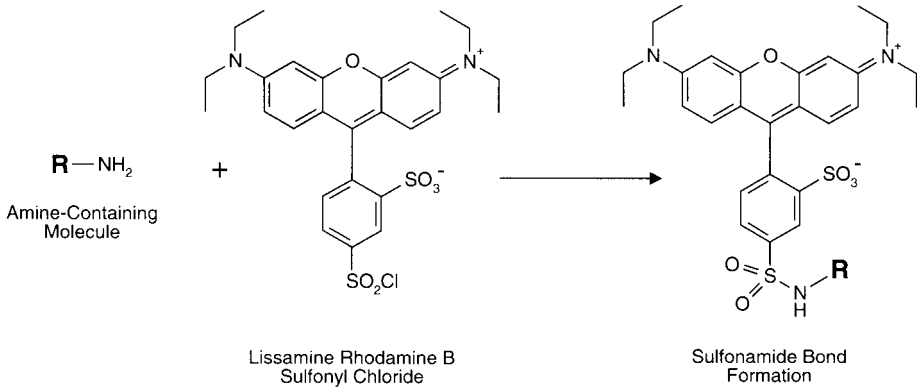


Figure 9.16 Lissamine rhodamine B sulfonyl chloride reacts with amine-containing molecules to produce stable sulfonamide bonds.

3- and 5-carbon positions of the lower ring. Lissamine rhodamine B sulfonyl chloride is an amine-reactive reagent made by converting the No. 5 sulfonate group to a reactive sulfonyl halide. Reaction with proteins and other amine-containing molecules results in the formation of sulfonamide bonds (Figure 9.16). Lissamine is a trademark of Imperial Chemical Industries.

The spectral characteristics of protein conjugates made with Lissamine rhodamine B derivatives are of longer wavelength than those of tetramethylrhodamine—more toward the red region of the spectrum. In addition, modified proteins have better chemical stability and are somewhat easier to purify than those made from TRITC (discussed previously). Lissamine derivatives also make more photostable probes than the fluorescein derivatives (Section 1, this chapter).

Lissamine rhodamine B sulfonyl chloride is relatively insoluble in water, but may be dissolved in DMF prior to the addition of a small aliquot to an aqueous reaction. Do not dissolve in DMSO, as sulfonyl chlorides will readily react with this solvent (Boyle, 1966). The compound has a maximal absorptivity at 556 nm with an extremely high extinction coefficient of up to 93,000 $M^{-1}cm^{-1}$ (in methanol) in highly purified form. Its emission maximum occurs at 576 nm, emitting red luminescence.

A sulfonyl chloride rapidly reacts with amines on proteins and other molecules to form stable sulfonamide bonds. It also may react with tyrosine —OH groups, aliphatic alcohols, thiols, and imidazole groups (such as histidine side chains). Conjugates of sulfonyl chlorides with sulfhydryls and imidazole rings are unstable, while esters formed with alcohols are subject to nucleophilic displacement (Nillson and Mosbach, 1984; Scouten and Van der Tweel, 1984). The only stable derivative with proteins therefore is the sulfonamide, formed by reaction with ε-lysine and N-terminal amines. Optimal conditions for coupling are non-amine-containing buffers in the pH range of 9–10. Phosphate, bicarbonate, or borate buffers are recommended for the modification reaction. Avoid the presence of other nucleophiles that can cross-react with the sulfonyl chloride (e.g., amine-containing components or sulfhydryl-containing reducing agents). In aqueous solutions, hydrolysis is a competing reaction, but occurs more slowly with sulfonyl halides than with the corresponding acid chlorides of carboxylate groups. Unreacted, hydrolyzed probe is the water-soluble sulforhodamine B fluorophore which is easily removed by gel filtration or dialysis.

Lissamine rhodamine B sulfonyl chloride has been used in numerous applications, including multiple-labeling techniques in microscopy (Wessendorf, 1990), for confocal microscopy

techniques (Tsien and Waggoner, 1990), in the study of fibronectin receptors (Duband *et al.*, 1988), for investigations into microtubule and intermediate filament association (Geiger and Singer, 1980), for the labeling of glycoconjugates (Wilchek *et al.*, 1980), for studying regulation of the Na^+/K^+-ATPase system (Sipe *et al.*, 1991), and for investigations into the redox potential within mitochondria (Chazotte and Hackenbrock, 1991). The dye also has been used to study the shape of giant unilamellar vesicles as model plasma membranes (Gudheti *et al.*, 2007), to investigate the activity of xyloglucan xylogucosyl transferase (Hrmova *et al.*, 2007), and the switching of neurofilaments between mobile and stationary states (Trivedi *et al.*, 2007). Hundreds of additional biological studies have used this fluorescent probe.

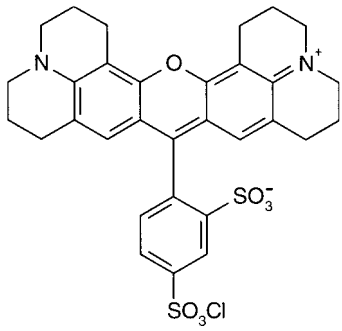
The following protocol is a general guide for labeling biological macromolecules with Lissamine rhodamine B sulfonyl chloride. Optimization of the fluorophore incorporation level (*F/P* ratio) may have to be done for specific labeling experiments.

Protocol

1. Dissolve the amine-containing macromolecule to be labeled (i.e., a protein) in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.0, at a concentration of 1–5 mg/ml.
2. Dissolve Lissamine rhodamine B sulfonyl chloride (Invitrogen) in DMF at a concentration of 1–2 mg/ml. Protect from light and use immediately.
3. In a darkened lab and with gentle mixing, slowly add 50–100 µl of the fluorophore solution to the protein solution.
4. React for 1 hour at room temperature in the dark.
5. Remove excess fluorophore by gel filtration using a desalting resin or by dialysis.

Texas Red Sulfonyl Chloride

Texas Red sulfonyl chloride is the active halogen derivative of sulforhodamine 101. This important derivative of the basic rhodamine molecule possesses dual aliphatic rings off the upper-ring nitrogens and sulfonate groups on the No. 3 and 5 carbon atoms of its lower-ring component. The sulfonyl chloride group can react with primary amines in proteins and other molecules to form stable sulfonamide bonds (Figure 9.17). The group, however, can hydrolyze



Texas Red Sulfonyl Chloride
MW 577
Excitation = 556 nm
Emission = 576 nm
ε at 556 nm = 93,000 $M^{-1}cm^{-1}$

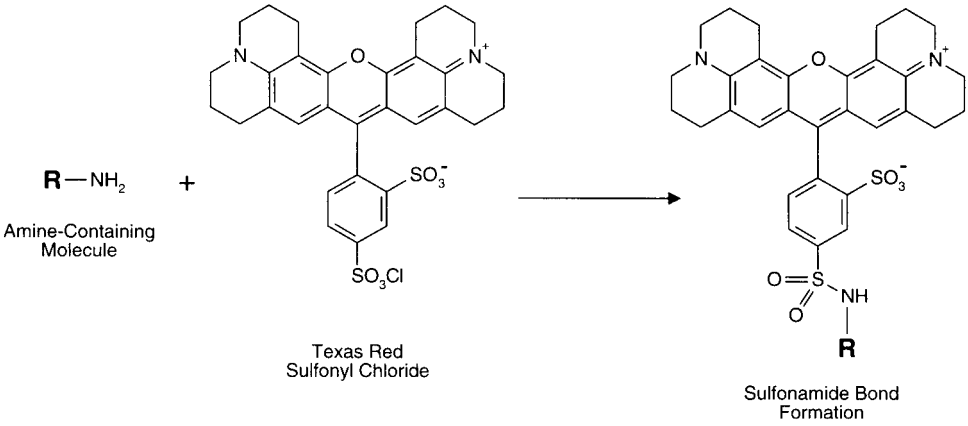


Figure 9.17 Texas Red sulfonyl chloride can be used to label amine-containing molecules through sulfonamide bond formation.

in the presence of moisture. For this reason, only fresh Texas Red sulfonyl chloride should be used for modification experiments.

The intense Texas Red fluorophore has a QY that is inherently higher than the tetramethylrhodamine or Lissamine rhodamine B derivatives. Texas Red's luminescence is shifted maximally into the red region of the spectrum, and its emission peak only minimally overlaps with that of fluorescein. This makes Texas Red derivatives among the best choices of labels for use in double-staining techniques.

Texas Red sulfonyl chloride has a maximal excitation at 589 nm and a maximum emission at 615 nm when dissolved in methanol. The extinction coefficient of the compound dissolved in acetonitrile is 85,000 $M^{-1}cm^{-1}$ at 596 nm. The only disadvantage of this fluorophore is its poor excitation by the standard argon laser at 488 nm. However, since both Texas Red and fluorescein are weakly excited by an argon laser at 514 nm, it makes them fairly good pairs for use in laser confocal microscopy or flow cytometry (Mossberg and Ericsson, 1990). The fluorophore is particularly appropriate for excitation by the 568 nm line produced by an argon–krypton mixed laser used on some confocal devices. Compared to other rhodamine derivatives, Texas Red fluorophores display low background in staining techniques and are among the most photostable probes available.

Texas Red sulfonyl chloride is soluble in DMF or acetonitrile and may be dissolved as a concentrated stock solution in either solvent prior to the addition of a small aliquot to an aqueous reaction medium. Avoid the use of DMSO, as sulfonyl chlorides react with this solvent (Boyle, 1966). The solid and all solutions made from it must be protected from light to avoid photodecomposition. Prepare the stock solution immediately before use.

A sulfonyl chloride group rapidly reacts with amines in the pH range of 9–10 to form stable sulfonamide bonds. Under these conditions, it also may react with tyrosine —OH groups, aliphatic alcohols, thiols, and histidine side chains. Conjugates of sulfonyl chlorides with sulfhydryls and imidazole rings are unstable, while esters formed with alcohols are subject to nucleophilic displacement (Nillson and Mosbach, 1984; Scouten and Van der Tweel, 1984). The only stable derivative with proteins therefore is the sulfonamide, formed by reaction with ε-lysine

and N-terminal amines. For coupling, the reaction media should use only non-amine-containing buffers, such as phosphate, borate, or bicarbonate (avoid Tris, imidazole, or glycine).

A suggested protocol on the use of this fluorescent probe is described below. Optimization may be necessary to achieve the best level of fluorescent modification (*F/P* ratio) for a particular application.

Protocol

1. Dissolve the protein or macromolecule to be labeled in 0.1 M sodium carbonate, pH 9.0, at a concentration of 1–5 mg/ml.
2. Dissolve Texas Red sulfonyl chloride (Thermo Fisher, Invitrogen) in acetonitrile at a concentration of 20 mg/ml. Prepare fresh and protect from light. Use a fume hood for all operations using organic solvents.
3. In subdued lighting conditions, add 50 µl of the Texas Red sulfonyl chloride solution to each ml of the protein solution. Mix well.
4. React for 1 hour at room temperature.
5. Remove excess fluorophore and reaction by-products by gel filtration using a desalting resin or by dialysis.

Determine the level of fluorophore incorporation (the *F/P* ratio) by measuring the absorbance of the labeled protein at 520 and 280 nm. Labeled proteins having a 520 nm/280 nm ratio of absorbency of 0.3–0.8 should perform well in most applications (Titus *et al.*, 1982).

At the time of this writing, over 15,000 references cited the use of Texas Red for labeling biological molecules or in bioconjugate detection applications.

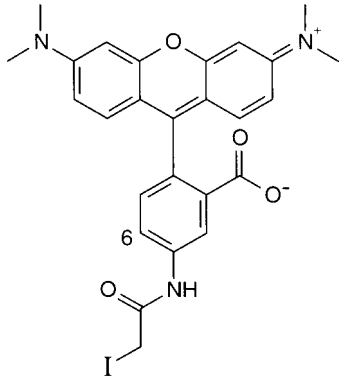
Sulfhydryl-Reactive Rhodamine Derivatives

Rhodamine derivatives containing a sulfhydryl-reactive group off the lower-ring structure are available to direct the modification reaction to more limited sites on target molecules. Coupling through sulfhydryls instead of amines can help to avoid active centers in proteins, thus preserving activity in the fluorescent probe complex. Sulfhydryl reaction sites can be naturally available through free cysteine side chains, generated by reduction of disulfides, or created by the use of thiolation reagents (Chapter 1, Section 4.1).

Tetramethylrhodamine-5-(and 6)-iodoacetamide

The iodoacetamido derivatives of tetramethylrhodamine possess a sulfhydryl-reactive iodoacetyl group (Chapter 1, Section 4.2 and Chapter 2, Section 2.1) at either the 5- or 6-carbon position on their lower ring. The isomers are commercially available only in mixed form, but some reactivity and specificity differences between the purified 5- and 6-derivatives toward various sulfhydryl sites in proteins may be observed (Ajtai *et al.*, 1992) (Invitrogen).

The iodoacetyl group of both isomers reacts with sulfhydryls under slightly alkaline conditions to yield stable thioether linkages (Figure 9.18). They do not react with unreduced disulfides in cystine residues or with oxidized glutathione (Gorman *et al.*, 1987). The thioether bonds are hydrolyzed under conditions necessary for complete protein hydrolysis prior to amino acid analysis.



Tetramethylrhodamine-
5-iodoacetamide
MW 569
Excitation = 540 nm
Emission = 567 nm
ε at 540 nm = 76,000 $M^{-1}cm^{-1}$

Care must be taken to protect these reagents from light, not only to maintain the fluorescent yield of the rhodamine derivative, but also to protect the iodoacetyl group from light-catalyzed breakdown. Tetramethylrhodamine-5-(and-6)-iodoacetamide is soluble in DMF and DMSO. Concentrated stock solutions may be prepared in these solvents prior to addition of a small aliquot to an aqueous reaction mixture. Protect solutions from light by wrapping in aluminum foil and working in subdued light. Quenching reactions with cysteine, glutathione, or mercaptosuccinic acid will sometimes facilitate removal of unconjugated fluorophore by dialysis or gel filtration.

The spectral properties of these derivatives are similar to native rhodamine. The excitation maximum occurs at about 543 nm and its emission peak at 567 nm, producing light in the orange-red region of the spectrum. The extinction coefficient of tetramethylrhodamine-5-(and-6)-iodoacetamide in methanol at its wavelength of maximum absorptivity, 542 nm, is 81,000 $M^{-1}cm^{-1}$.

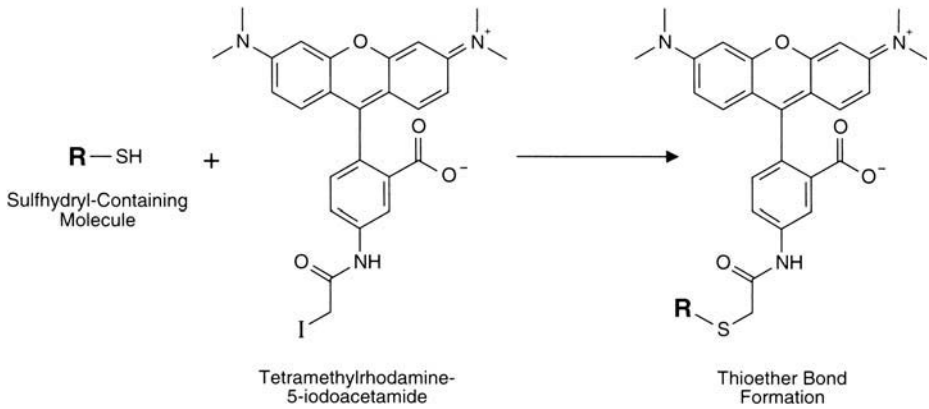


Figure 9.18 This iodoacetamide derivative of tetramethylrhodamine can be used to label sulfhydryl groups via thioether bond formation.

The fluorescent probe has been used extensively to label numerous proteins and other biomolecules, including actin (Glacy, 1983; Wang, 1985; Meige and Wang, 1986), myosin light chains (Mittal *et al.*, 1987), α-actin (Simon and Taylor, 1988; Stickel and Wang, 1988), blood coagulation factor Va (Isaacs *et al.*, 1986), and histones (Murphy *et al.*, 1982). The dye also has been used to study conformational changes in proteins (Heuck *et al.*, 2007), to study the binding region of protein C on factor Va (Yegneswaran *et al.*, 2007), and how flavonoids affect actin functions (Boehl *et al.*, 2007). Hundreds of additional publications cite the use of this dye for various biological detection applications.

The following protocol for labeling proteins with tetramethylrhodamine-5-(and-6)-iodoacetamide represents a general guideline. The procedure should be optimized for each macromolecule being labeled to obtain the best *F/P* ratio to produce intense fluorescence and high activity in the final complex.

Protocol

1. Prepare a 20 mM tetramethylrhodamine-5-(and-6)-iodoacetamide solution by dissolving 11.3 mg/ml of DMF. Prepare fresh and protect from light.
2. Dissolve the protein to be modified at a concentration of 5–10 mg/ml in 50 mM sodium phosphate, pH 7.5.
3. Slowly add 25–50 µl of the tetramethylrhodamine-5-(and-6)-iodoacetamide solution to each ml of the protein solution while mixing.
4. React for 2 hours at 4°C in the dark.
5. Remove excess reactant and reaction by-products by gel filtration using a desalting resin or by dialysis.

Aldehyde/Ketone and Cytosine-Reactive Rhodamine Derivatives

Hydrazide groups can be coupled directly to aldehydes and ketones to form relatively stable hydrazone linkages (Chapter 2, Section 5.1). Two rhodamine derivatives are commonly available that contain a sulfonyl hydrazine group off their No. 5 carbon on the lower-ring structure (Invitrogen). They are based on the Lissamine and Texas Red structures and may be used to label aldehyde- or ketone-containing molecules with an intensely fluorescent probe. Although most biomolecules don't contain aldehyde or ketone groups in their native state, carbohydrates, glycoproteins, RNA, and other molecules containing sugar residues (or diols) can be oxidized with sodium periodate to produce reactive formyl groups. The use of modification reagents which generate aldehydes upon coupling to a molecule also can be used to produce a hydrazide-reactive site (Chapter 1, Section 4.4).

DNA and RNA may be modified with hydrazide-reactive probes by reacting their cytosine residues with bisulfite to form reactive sulfone intermediates. These derivatives undergo transamination to couple with hydrazide- or amine-containing probes (Draper and Gold, 1980) (Chapter 27, Section 2.1).

Lissamine Rhodamine B Sulfonyl Hydrazine

Lissamine rhodamine B sulfonyl hydrazine is a hydrazide derivative of sulforhodamine B that can spontaneously react with aldehyde- or ketone-containing molecules to form a covalent,

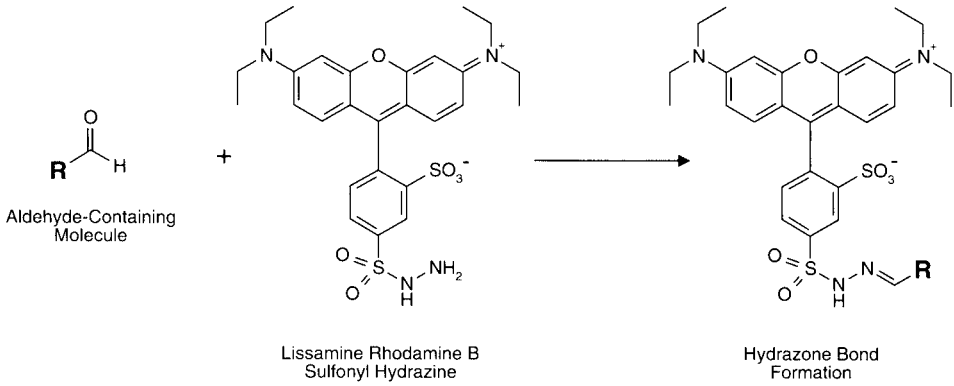
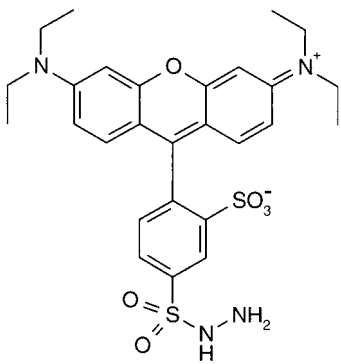


Figure 9.19 Lissamine rhodamine B sulfonyl hydrazine can react with aldehyde groups to form hydrazone linkages.

hydrazone linkage (Figure 9.19). It also can be used to label cytosine residues in DNA or RNA by use of the bisulfite activation procedure (Chapter 27, Section 2.1). The resulting fluorescent derivative exhibits an excitation maximum at a wavelength of 556 nm and a maximal emission wavelength of 580 nm when dissolved in methanol. In the same solvent, the compound has an extinction coefficient of approximately 75,000 $M^{-1}cm^{-1}$.



Lissamine Rhodamine B
Sulfonyl Hydrazine
MW 573
Excitation = 560 nm
Emission = 585 nm
ε at 560 nm = 95,000 $M^{-1}cm^{-1}$

Lissamine rhodamine B sulfonyl hydrazine is soluble in DMF. The reagent may be dissolved in this solvent as a concentrated stock solution before adding a small aliquot to an aqueous reaction medium. The compound itself and all solutions made with it should be protected from light to avoid decomposition of its fluorescent properties.

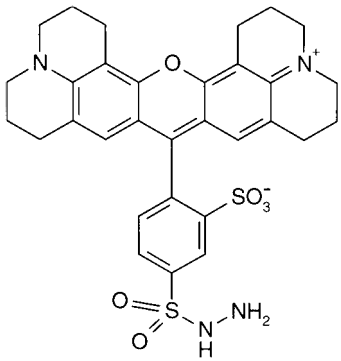
Generalized protocols for the use of hydrazine probes reactive toward aldehyde residues can be found in Section 1, this chapter. These procedures are directed at the labeling of cell-surface

glycoproteins or glycoproteins in solution. Substitution of Lissamine rhodamine B sulfonyl hydrazine for the fluorescein-5-thiosemicarbazide reagent described in that section can be done without difficulty. Some optimization may be necessary to achieve the best level of fluorescent modification for each particular application.

Texas Red Hydrazide

Texas Red hydrazide is a derivative of Texas Red sulfonyl chloride made by reaction with hydrazine (Invitrogen). The result is a sulfonyl hydrazine group on the No. 5 carbon position of the lower-ring structure of sulforhodamine 101. The intense Texas Red fluorophore has a QY that is inherently higher than either the tetramethylrhodamine or Lissamine rhodamine B derivatives of the basic rhodamine molecule. Texas Red's luminescence is shifted maximally into the red region of the spectrum, and its emission peak only minimally overlaps with that of fluorescein. This makes derivatives of this fluorescent probe among the best choices of labels for use in double-staining techniques.

The hydrazide derivative can be used to modify aldehyde- or ketone-containing molecules, including cytosine residues using the bisulfite activation procedure described in Chapter 27, Section 2.1. The sulfonyl hydrazine group of Texas Red hydrazide reacts with aldehydes or ketones in target molecules to form hydrazone bonds (Figure 9.20). Carbohydrates and glycoconjugates can be specifically labeled at the polysaccharide portion if the required aldehydes are first formed by periodate oxidation or another such method (Chapter 1, Section 4.4).



Texas Red Hydrazide
MW 621
Excitation = 580 nm
Emission = 604 nm
ε at 580 nm = 80,000 $M^{-1}cm^{-1}$

Texas Red hydrazide has a maximal excitation wavelength of 580 nm and a maximum emission at 604 nm when dissolved in methanol. Its extinction coefficient in the same solvent is 80,000 $M^{-1}cm^{-1}$ at 580 nm. The only disadvantage of this fluorophore is its poor excitation by an argon laser at 488 nm. However, since both Texas Red and fluorescein are weakly excited by an argon laser at 514 nm, it makes them fairly good pairs for use in laser confocal microscopy or flow cytometry (Mossberg and Ericsson, 1990). The fluorophore is particularly

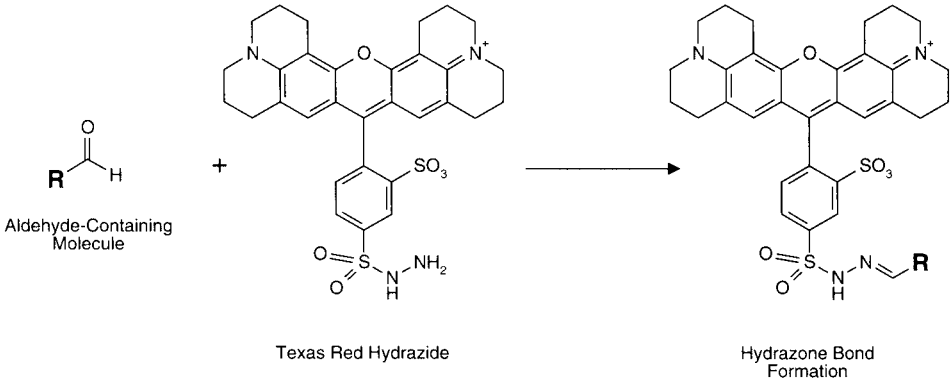


Figure 9.20 Texas Red hydrazide reacts with aldehydes to create hydrazone bonds.

appropriate for excitation by the 568 nm line produced by an argon–krypton mixed laser used on some confocal devices. White light illumination also can be used to excite the dye, while its emission is detected using an appropriate filter. Compared to other rhodamine derivatives, Texas Red fluorophores display low background in staining techniques and are among the most photostable probes available.

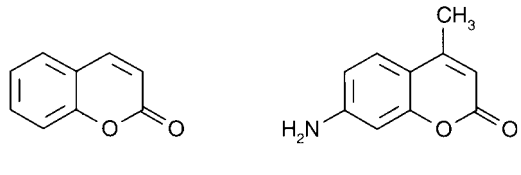
Texas Red hydrazide is soluble in DMF and may be dissolved as a concentrated stock solution in this solvent prior to the addition of a small aliquot to an aqueous reaction medium. The solid and all solutions made from it must be protected from light to avoid photo-decomposition. Prepare the stock solution fresh immediately before use. A suggested protocol on the use of this fluorescent probe may be obtained by following the method outlined for fluorescein-5-thiosemicarbazide in Section 1 of this chapter. Optimization may be necessary to achieve the best level of fluorescent modification (*F*/*P* ratio) for a particular application.

3. Coumarin Derivatives

Coumarin (2*H*-1-benzopyran-2-one) is a naturally occurring substance found in tonka beans, lavender oil, and in sweet clover (Merck Index **11**: 2563) (Figure 9.21). Many of its derivatives are highly fluorescent compounds that are widely studied (Schimitschek *et al*., 1974; Ernsting *et al*., 1982; Jones *et al*., 1984, 1985; Eschrich and Morgan, 1985; Baranowska-Kortylewicz and Kassis, 1993a, b). The 7-amino-4-methylcoumarin derivatives have excellent fluorescent properties useful for labeling biological molecules with a detectable tag (Uchino *et al*., 1979; Bos, 1981). Particularly, the 3-acetic acid derivative of this molecule, known as AMCA, provides a carboxylate group from which to create easily reactive groups suitable for coupling to proteins and other molecules.

Aminomethylcoumarin derivatives possess intense fluorescent properties within the blue region of the visible spectrum. Their emission range is sufficiently removed from other common fluorophores that they are excellent choices for double-labeling techniques. In fact, coumarin fluorescent probes are very good donors for excited-state energy transfer to fluoresceins.

The following sections describe the most popular derivatives of aminomethylcoumarin used to label proteins and other biological macromolecules.



Coumarin
(2H-1-benzopyran-2-one)

7-Amino-4-methyl-
coumarin

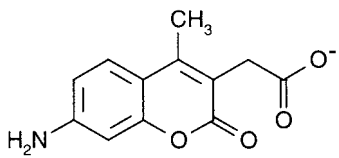
Figure 9.21 The basic structural characteristics of coumarin fluorophores.

Amine-Reactive Coumarin Derivatives

Three main forms of amine-reactive AMCA probes are commonly available. One of them is simply the free acid form of AMCA, which can be used to couple to amine-containing molecules using the carbodiimide reaction (Chapter 3, Section 1.1). The other two are active-ester derivatives of AMCA—the water-insoluble NHS ester and the water-soluble sulfo-NHS ester forms—both of which spontaneously react with amines to create stable amide linkages. All of them react under mild conditions with primary amines in proteins and other molecules to form highly fluorescent derivatives.

AMCA

AMCA, or 7-amino-4-methylcoumarin-3-acetic acid, is a fluorescent probe that exhibits a spectacular blue fluorescence (Khalfan *et al.*, 1986) (Thermo Fisher). AMCA absorbs light at a wavelength of 345 nm and luminesces in the range of 440–460 nm. Its emission wavelength is in a region that doesn't overlap with the emission spectra of other major fluorescent probes. This makes double-staining techniques particularly effective with this fluorophore. AMCA also has pronounced stability toward photobleaching, retaining its full fluorescence more than 3 times longer than fluorescein-based probes. AMCA derivatives and labeled molecules fluoresce with a bright blue color upon excitation. This color is easily visualized using fluorescent microscopes or imagers. The blue emission color avoids problems of autofluorescence associated with high background. In addition, the large Stoke's shift of the molecule minimizes interference from Rayleigh light scatter effects during excitation. The fluorescent intensity of AMCA is not affected by changes in pH in the range of 3–10. This is in marked contrast to other fluorescent probes, such as fluorescein, which display considerable variability in their emission spectra with pH.

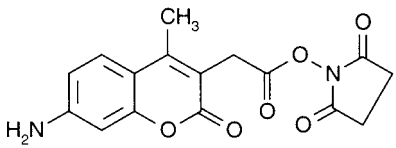


AMCA
7-Amino-4-methyl-
coumarin-3-acetic acid
MW 233
Excitation = 345-350 nm
Emission = 440-460 nm

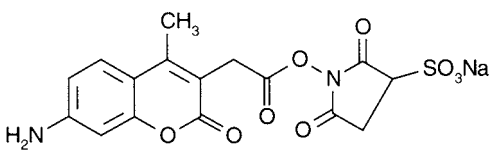
AMCA may be coupled to amine-containing molecules through the use of the carbodiimide reaction using EDC (Chapter 3, Section 1.1). EDC will activate the carboxylate on AMCA to a highly reactive *o*-acylisourea intermediate. Attack by a nucleophilic primary amine group results in the formation of an amide bond (Figure 9.22). Derivatization of AMCA off its carboxylate group causes no major effects on its fluorescent properties. Thus, proteins and other macromolecules may be labeled with this intensely blue probe and easily detected by fluorescence microscopy and other techniques.

AMCA-NHS and AMCA-Sulfo-NHS

AMCA-NHS, succinimidyl-7-amino-4-methylcoumarin-3-acetic acid, is an amine-reactive derivative of AMCA containing an NHS ester on its carboxylate group (Thermo Fisher). The result is reactivity directed toward amine-containing molecules, forming amide linkages with the AMCA fluorophore (Figure 9.23). Proteins labeled with AMCA show little-to-no effect on the isoelectric point of the molecule.



AMCA–NHS
Succinimidyl-7-amino-
4-methylcoumarin-3-acetic acid
MW 330



AMCA–Sulfo-NHS
Sulfosuccinimidyl-7-amino-
4-methylcoumarin-3-acetic acid
MW 431

Reaction of AMCA-NHS with proteins proceeds efficiently in the pH range of 7–9. Avoid buffers containing amines which can compete in the coupling reaction, such as Tris or glycine, and avoid imidazole buffers since they promote hydrolysis of the NHS ester. AMCA-NHS is relatively insoluble in aqueous buffers. The compound must be first dissolved in organic solvent prior to adding a small aliquot to the reaction mixture. A concentrated stock solution may be prepared in DMSO and stored up to 2 weeks refrigerated or frozen without loss of activity. The solid and all solutions of AMCA-NHS should be protected from light to avoid photobleaching of the fluorophore.

AMCA-sulfo-NHS is an analog of AMCA-NHS which contains a sulfonate group on its NHS ring (Thermo Fisher). The negative charge of this group provides enough polarity to

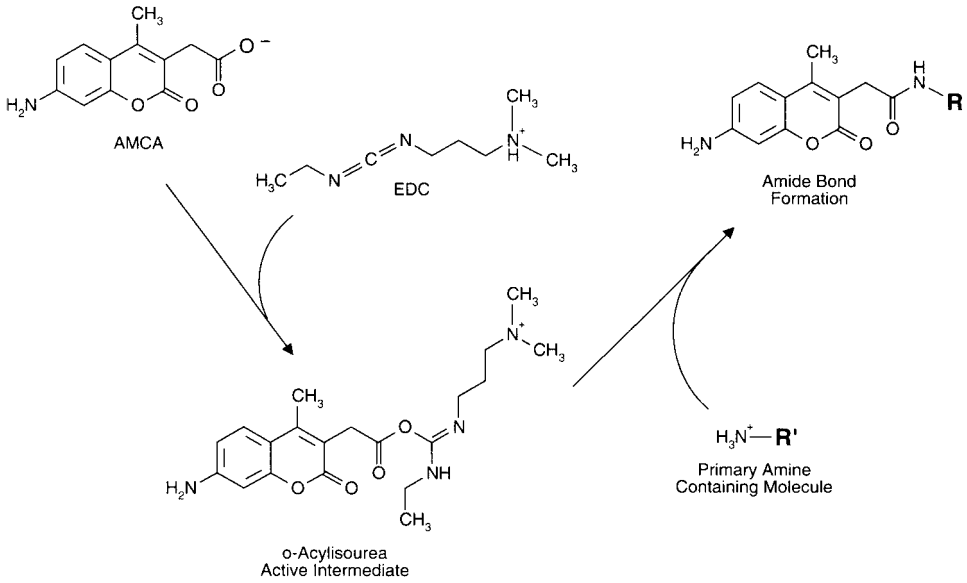


Figure 9.22 AMCA may be linked to amine-containing molecules through its carboxylate group using a carbodiimide reaction with EDC.

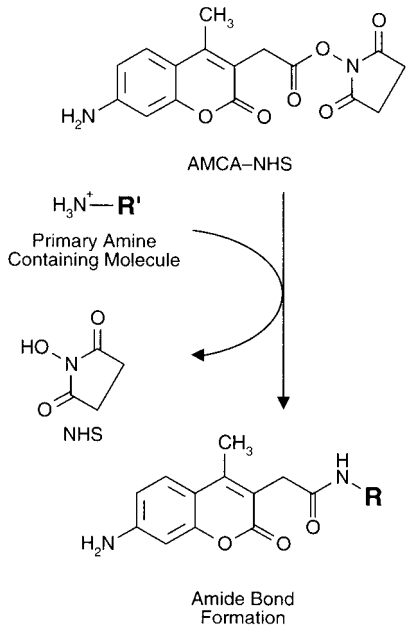


Figure 9.23 AMCA-NHS reacts with amines to form amide bonds.

promote water solubility for the entire reagent. The reactivity and properties of AMCA-sulfo-NHS are identical to those of AMCA-NHS.

Preparing an optimal fluorescent conjugate is largely dependent upon the degree of modification with the label. The following protocol is generalized for the labeling of a protein with AMCA-NHS. For particular labeling experiments, it is often necessary to vary the amount of fluorophore added to the reaction mixture to obtain the best combination of protein activity and fluorescent intensity in the conjugate. Too much label and nonspecific binding or fluorescent quenching may result; too little label and the complex will not possess enough fluorescent intensity to be sufficiently detectable.

Protocol

1. Dissolve the protein to be modified in 50 mM sodium borate, pH 8.5, at a concentration of 10 mg/ml. Other buffers may be used for an NHS ester reaction, including 0.1 M sodium phosphate, pH 7.5 (Chapter 2, Section 1.4).
2. Dissolve AMCA-NHS (Thermo Fisher) in DMSO at a concentration of 2.6 mg/ml. Protect from light.
3. In subdued lighting conditions, slowly add 50–100 µl of the AMCA-NHS stock solution to each ml of the protein solution, with gentle mixing.
4. React for 1 hour at room temperature in the dark.
5. Remove excess reagent and reaction by-products by gel filtration using a desalting resin. The sample volume should be no more than about 5–8 percent of the column volume.

The *F/P* ratio of the purified, labeled protein may be determined by measuring the absorbance at 345 and 280 nm. Ratios between 0.3 and 0.8 usually produce labeled molecules having acceptable levels of fluorescent intensity and good retention of protein activity. AMCA-labeled proteins may be lyophilized without significant loss of fluorescence. The addition of bovine serum albumin (15 mg/ml) or another such stabilizer is often necessary to retain solubility of the freeze-dried, labeled protein after reconstitution.

Sulfhydryl-Reactive Coumarin Derivatives

Two aminomethylcoumarin derivatives are available for labeling sulfhydryl-containing molecules. The ability to label —SH groups in proteins provides a means of directing the modification reaction to a limited number of sites, possibly avoiding active centers or binding regions better than when using amine-reactive probes. The first sulfhydryl-reactive probe discussed in this section makes use of a pyridyl disulfide group on the AMCA derivative. The second probe is an iodoacetyl compound made from a diethyl and aminophenyl derivative of the basic aminomethylcoumarin structure.

AMCA-HPDP

AMCA-HPDP is *N*-[6-(7-amino-4-methylcoumarin-3-acetamido)hexyl]-3′-(2′-pyridyldithio) propionamide. It is formed from AMCA plus a 1,6-diaminohexyl spacer off the carboxylate that has been additionally modified at its terminal end with SPDP (Chapter 5, Section 1.1).

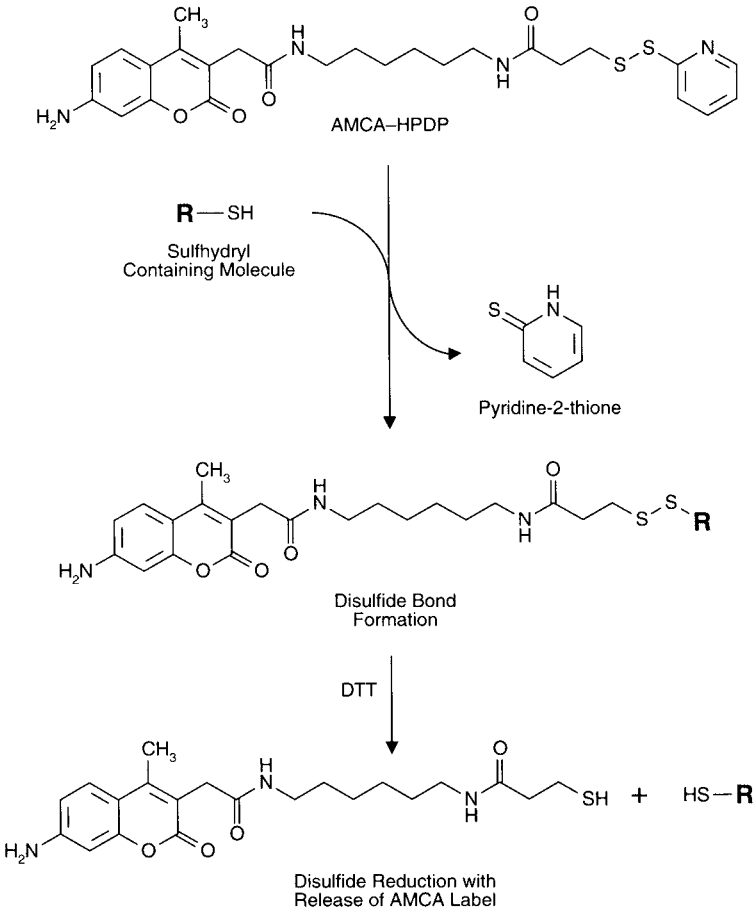
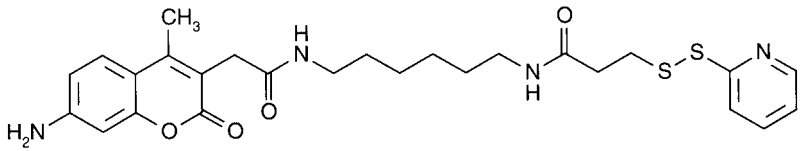


Figure 9.24 AMCA-HPDP reacts with sulfhydryl groups through its pyridyl disulfide end to form reversible disulfide bonds.

The result is a long spacer arm terminating in a pyridyl disulfide group reactive toward free sulfhydryl residues. The reaction of this group with a thiol creates a disulfide bond between the AMCA fluorophore and the molecule being modified. Thus, the fluorescent tag can be specifically cleaved by reduction with DTT or other disulfide reducing agents (Figure 9.24).



AMCA–HPDP
N-[6-(7-amino-4-methylcoumarin-
3-acetamido)hexyl]-3'-(2'-pyridyldithio)propionamide
Excitation = 345 nm
Emission = 440-460 nm

The required sulfhydryl residues can be naturally occurring on a protein, created by reduction of cystine crosslinks or by thiolation (Chapter 1, Section 4.1). For the labeling of antibody molecules, mild reduction with 2-mercaptoethylamine, DTT, or tris(2-carboxyethyl)phosphine (TCEP) results in free sulfhydryl groups in the hinge region. Labeling in this area is advantageous to direct the modification away from antigen binding regions. Sulfhydryl residues also may be created on oligonucleotides without difficulty (Chapter 27, Section 2.2).

Although the reaction of a sulfhydryl-containing molecule with AMCA-HPDP results in the release of the chromogenic leaving group, pyridine-2-thione, using it to quantify the extent of modification may be difficult, because it absorbs at 343 nm, which is in the same region as AMCA itself (345 nm). The emission range of the AMCA probe is about 440–460 nm, in the blue region of the spectrum.

The following protocol is a suggested method for labeling a protein with AMCA-HPDP. It is assumed that the presence of a sulfhydryl on the protein has been documented or created. The reaction conditions can be carried out in a variety of buffers between pH 6 and 9. Avoid the presence of extraneous sulfhydryl-containing compounds (such as disulfide reductants) that will compete in the reaction. The inclusion of EDTA in the modification buffer prevents metal-catalyzed sulfhydryl oxidation. Optimization for a particular labeling experiment should be done to obtain the best level of fluorophore incorporation.

Protocol

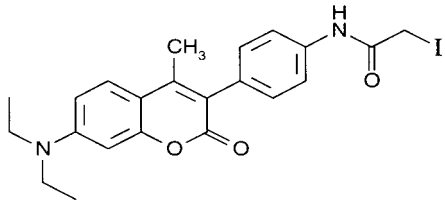
1. Dissolve the sulfhydryl-containing protein to be labeled in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2, at a concentration of 10 mg/ml.
2. Dissolve AMCA-HPDP in DMSO at a concentration of 0.5 mg/ml. Protect from light.
3. In subdued lighting conditions, add 50–100 µl of the AMCA-HPDP stock solution to each ml of sulfhydryl-containing protein solution. Mix.
4. React for 1 hour at room temperature in the dark with occasional mixing.
5. Remove excess fluorophore and reaction by-products by gel filtration using a desalting resin.

To determine the *F/P* ratio of the labeled protein, measure the absorbance of the purified preparation at 345 nm and 280 nm. Ratios of 345 nm/280 nm within the range of 0.3–0.8 usually result in fluorescent conjugates with a good balance of high-intensity luminescence, low nonspecific binding, and excellent retention of biological activity within the protein component.

DCIA

DCIA is 7-diethylamino-3-[(4′-(iodoacetyl)amino)phenyl]-4-methylcoumarin, a derivative of the basic aminomethylcoumarin structure that contains a sulfhydryl-reactive iodoacetyl group and a diethyl substitution on its amine. This particular coumarin derivative is among the most fluorescent UV-excitable iodoacetamide probes available (Sippel, 1981) (Invitrogen).

The iodoacetyl group of DCIA reacts with sulfhydryls under slightly alkaline conditions to yield stable thioether linkages (Figure 9.25). They do not react with unreduced disulfides in cystine residues or with oxidized glutathione (Gorman *et al.*, 1987).



DCIA
7-Diethylamino-3-((4'-(iodoacetyl)-amino)phenyl)-4-methylcoumarin
MW490
Excitation = 382 nm
Emission = 472 nm
ε at 382 nm = 33,000 $M^{-1}cm^{-1}$

Care must be taken to protect iodoacetyl reagents from light, not only to maintain the fluorescent yield of the coumarin component, but also to protect the iodoacetyl group from light-catalyzed breakdown. DCIA is soluble in DMF and DMSO. Concentrated stock solutions may be prepared in either solvent prior to addition of a small aliquot to a reaction mixture. Protect solutions from light by wrapping vessels in aluminum foil and working in subdued light.

The spectral properties of this fluorophore are similar to those of other coumarin derivatives. The excitation maximum occurs at about 382 nm and its emission peak at 472 nm, producing light in the blue region of the spectrum. The extinction coefficient of DCIA at its wavelength of maximum absorbance, 382 nm, is 33,000 $M^{-1}cm^{-1}$ (in methanol).

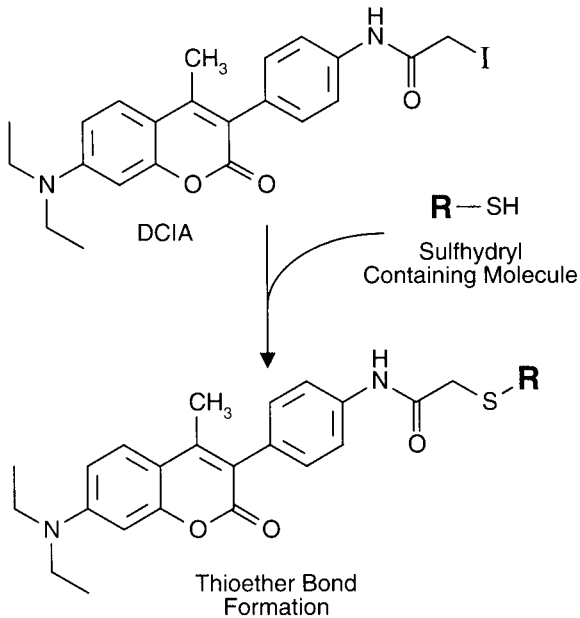


Figure 9.25 DCIA can modify sulfhydryl groups through its iodoacetamide group to form thioether linkages.

DCIA has been used to label numerous proteins and other biomolecules, including phospholipids (Silvius *et al.*, 1987), to study the interaction of mRNA with the 30S ribosomal subunit (Czworkowski *et al.*, 1991), in the investigation of cellular thiol components by flow cytometry (Durand and Olive, 1983), in the detection of carboxylate compounds using peroxyoxalate chemiluminescence (Grayeski and DeVasto, 1987), and for general sulfhydryl labeling (Sippel, 1981).

A general protocol for the use of DCIA for fluorescently labeling proteins that contain sulfhydryl residues may be obtained by following the method discussed for AMCA-HPDP (previous section). After purification of the labeled protein, the *F/P* ratio of fluorophore incorporation may be determined by measuring its 382 nm/280 nm absorbance ratio.

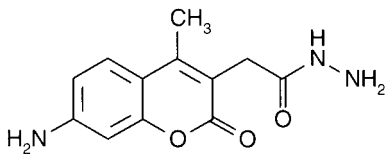
Aldehyde- and Ketone-Reactive Coumarin Derivatives

Hydrazide groups can be coupled directly to aldehyde and ketone groups to form relatively stable hydrazone linkages (Chapter 2, Section 5.1). One AMCA derivative is commonly available that contains a hydrazine group modification on its carboxylate. Although most biomolecules don't contain aldehyde or ketone groups in their native state, carbohydrates, glycoproteins, RNA, and other molecules that contain sugar residues can be oxidized with sodium periodate to produce reactive formyl groups. The use of modification reagents which generate aldehydes upon coupling to a molecule also can be used to produce a hydrazide-reactive site (Chapter 1, Section 4.4).

DNA and RNA may be modified with hydrazide-reactive probes by reacting their cytosine residues with bisulfite to form reactive sulfone intermediates. These derivatives can undergo transamination reactions with hydrazide- or amine-containing probes to yield covalent bonds (Draper and Gold, 1980) (Chapter 27, Section 2.1).

AMCA-Hydrazide

AMCA-hydrazide is 7-amino-4-methylcoumarin-3-acetyl hydrazide, a hydrazine derivative off the carboxyl group of the basic AMCA molecule (Thermo Fisher). AMCA-based fluorophores are highly stable toward photobleaching. Molecules labeled with this probe retain their fluorescent intensity over 3 times longer than a fluorescein label when exposed to light. In addition, AMCA derivatives exhibit a large Stoke's shift of over 100 nm, thus they are minimally affected by Rayleigh scattering effects during excitation. The blue light emitted by these labels is in a region of the spectrum well removed from the emission characteristics of other major fluorescent probes. This means that double-staining techniques easily can be used with an AMCA label. AMCA also exhibits little luminescence dependency on pH over the range of 3–10.



AMCA–Hydrazide
7-Amino-4-methylcoumarin-
3-acetyl hydrazide
MW 247. 1
Excitation = 345 nm
Emission = 440–460 nm

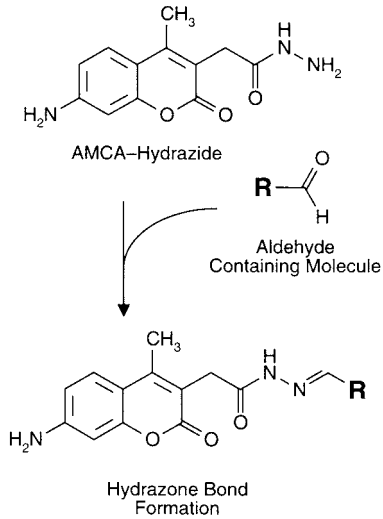


Figure 9.26 AMCA-hydrazide can be used to label aldehyde-containing molecules, such as periodate-oxidized carbohydrates.

The hydrazide derivative of AMCA can be used to modify aldehyde- or ketone-containing molecules, including cytosine residues using the bisulfite activation procedure described in Chapter 27, Section 2.1. AMCA-hydrazide reacts with these target groups to form hydrazone bonds (Figure 9.26). Carbohydrates and glycoconjugates can be labeled specifically at their polysaccharide portion if the required aldehydes are first formed by periodate oxidation or another such method (Chapter 1, Section 4.4).

AMCA-hydrazide has a maximal excitation wavelength of 345 nm and a maximum emission wavelength in the range of 440–460 nm. A solution of AMCA in PBS at a concentration of 16.7 ng/ml (71.61 nmoles/ml) gives an absorbance at 345 nm of about 1.28. This translates into a molar extinction coefficient at this wavelength of about 13,900 $M^{-1}cm^{-1}$. Different solvents and conditions may alter this value somewhat.

AMCA-hydrazide is soluble in DMSO or DMF and may be dissolved as a concentrated stock solution in either of these solvents prior to the addition of a small aliquot to an aqueous reaction medium. The solid and all solutions made from the fluorophore must be protected from light to avoid photo-decomposition. Prepare the stock solution fresh immediately before use. A suggested protocol on the use of this fluorescent probe may be obtained from the following method on the labeling of periodate-oxidized IgG. Optimization may be necessary to achieve the best level of fluorescent modification (*F/P* ratio) for a particular application.

Protocol

Oxidation of IgG Carbohydrate Residues with Sodium Periodate

1. Dissolve the antibody to be labeled in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of at least 10 mg/ml. The immunoglobulin must be glycosylated to work in this procedure.

2. Dissolve sodium periodate in water to a final concentration of 100 mM. Protect from light. Add 0.1 ml of this stock periodate solution to each ml of the antibody solution.
3. React for 15 minutes at room temperature, protected from light.
4. Quench the reaction by addition of 0.1 ml of glycerol per ml of reaction volume, mix, and then react for an additional 15 minutes. Remove excess reagents by gel filtration using a desalting column, and performing the chromatography using the phosphate buffer.
5. Adjust the concentration of IgG in the purified preparation to 1 mg/ml by the addition of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5.

Modification of Oxidized IgG with AMCA-Hydrazide

1. Dissolve AMCA-hydrazide in DMF at a concentration of 0.4 mg/ml. Protect from light.
2. Add 50–100 µl of the AMCA-hydrazide stock solution to each ml of the oxidized antibody solution.

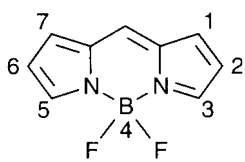
   Note: At a level of 50-µl probe addition, polyclonal human IgG will be modified at a level that gives an *F/P* ratio of about 0.113. Since the labeling occurs only at the oxidized carbohydrate sites, the fluorophore incorporation typically is less than that observed when using amine-reactive probes.
3. React for 30 minutes at room temperature in the dark.
4. Remove excess fluorophore by dialysis or gel filtration using a desalting resin. Protect the labeled immunoglobulin from light.

Determine the *F/P* ratio by measuring the absorbance at 345 and 280 nm.

4. BODIPY Derivatives

BODIPY fluorophores are a class of probes based on the fused, multi-ring structure, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene (Figure 9.27) (Invitrogen) (U.S. patent 4,774,339). This fundamental molecule can be modified, particularly at its 1, 3, 5, 7, and 8 carbon positions, to produce new fluorophores with different characteristics. The modifications cause spectral shifts in its excitation and emission wavelengths, and can provide sites for chemical coupling to label biomolecules.

The BODIPY derivatives typically have high extinction coefficients and excellent QY, often greater than 0.8. Their spectral characteristics are relatively insensitive to changes in pH.



The BODIPY Structure
4,4-Difluoro-4-bora-
3a,4a-diaza-s-indacene

Figure 9.27 The basic structure of BODIPY fluorophores.

Luminescent changes with shifts in pH usually are due to reconfiguration of a fluorophore's π-electron cloud if an atom on the ring system becomes protonated or unprotonated. Since the BODIPY structure lacks an ionizable group, alterations in pH have no effect on its spectral attributes.

The emission spectra of BODIPY derivatives normally display narrow bandwidths, providing intensely fluorescent labels for biomolecules. Unfortunately, they also have very small Stoke's shifts, typically on the order of only 10–20 nm. Excitation at the optimal wavelength may cause some interference in measurements at the emission wavelength due to light scattering or cross-over from the wide bandwidth of the excitation source. The dyes usually require excitation at sub-optimal wavelengths to prevent this problem.

The following sections discuss the major BODIPY derivatives that are reactive toward particular functional groups in proteins and other molecules.

Amine-Reactive BODIPY Derivatives

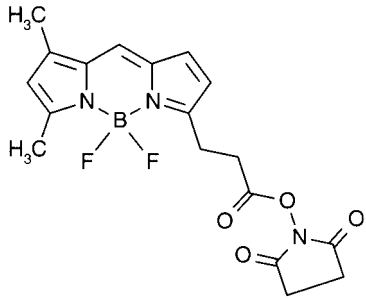
A number of BODIPY derivatives that contain reactive groups able to couple with amine-containing molecules are commonly available. The derivatives either contain a carboxylate group, which can be reacted with an amine in the presence of a carbodiimide to create an amide bond, or an NHS ester derivative of the carboxylate, which can react directly with amines to form amide linkages. The three discussed in this section are representative of this amine-reactive BODIPY family. The two NHS ester derivatives react under alkaline conditions with primary amines in molecular targets to form stable, highly fluorescent derivatives. The carboxylate derivative can be coupled to an amine using the EDC/sulfo-NHS reaction discussed in Chapter 3, Section 1.2.

The only disadvantage of using BODIPY fluorophores to label amines in macromolecules is the tendency for fluorescence quenching to occur if multiple sites on one molecule are modified. Especially with proteins, using an amine-reactive probe usually results in a number of sites being modified on each molecule. All fluorophores experience some quenching effect if the degree of substitution is high, because probe–probe interactions are possible that can transfer energy from an excited-state fluorophore to a ground-state fluorophore before luminescence occurs. BODIPY probes, however, are especially notorious for probe–probe quenching effects. For this reason, the manufacturer (Invitrogen) recommends that the amine-reactive BODIPY probes only be used to modify substances that have the potential for just one substitution per molecule. In this sense, BODIPY fluorophores are particularly well suited for tagging DNA probes at the 5′ end or lipid molecules on their head groups. Oligonucleotides modified to contain an amine on their 5′ phosphate group (Chapter 27, Section 2.1) are particularly good candidates for labeling with this fluorophore. Other BODIPY probes that contain reactivity toward non-amine functionalities such as sulfhydryls or polysaccharides may be more effective at labeling macromolecules like proteins, since these groups occur at more limited sites within the molecules and the modification level can be better controlled.

BODIPY FL C_3-SE

BODIPY FL C_3-SE is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester (Invitrogen). The derivatization to the base BODIPY molecule

results in fluorescent properties which mimic fluorescein in its emission wavelength. The molecule thus emits light in the green region of the spectrum. The NHS ester on its propionic side chain provides amine reactivity, resulting in amide bond linkages with modified molecules (Figure 9.28).



BODIPY FL C_3-SE
4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-sindacene-3-propionic acid, succinimidyl ester
MW 389
Excitation = 502 nm
Emission = 510 nm
ε at 502 nm = 77,000 $M^{-1}cm^{-1}$

This fluorophore has an excitation maximum at 502 nm and an emission maximum at 510 nm. The small Stoke's shift of only 8 nm creates some difficulty in discrete excitation without contaminating the emission measurement with scattered or overlapping light. The extinction coefficient of the molecule in methanol is about 77,000 $M^{-1}cm^{-1}$ at 502 nm.

BODIPY FL C_3-SE is insoluble in aqueous solution, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction. For aqueous reactions, a pH range of 7–9 is optimal. Avoid amine-containing buffers. The reaction also may be done in organic solvent.

Since BODIPY fluorophores are easily quenched if substitutions on a molecule exceed a 1:1 stoichiometry, modification of proteins with this fluorophore probably won't yield satisfactory

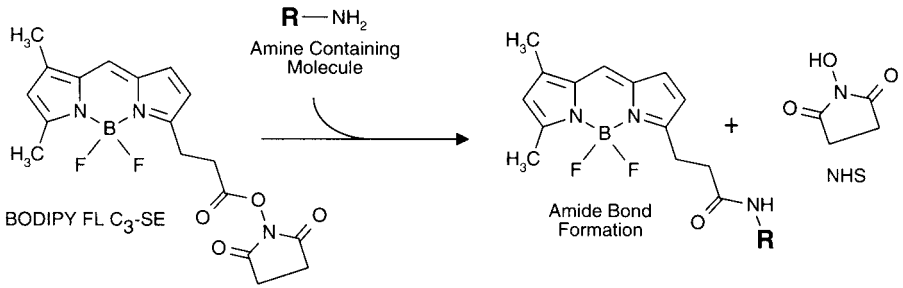
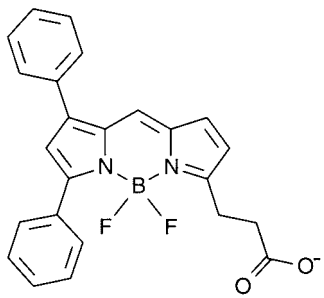


Figure 9.28 The side-chain NHS ester of this BODIPY derivative can be used to modify amine-containing molecules, forming amide bond linkages.

results. However, for labeling molecules which contain only one amine group, BODIPY FL C_3-SE will give intensely fluorescent derivatives.

BODIPY 530/550 C_3

BODIPY 530/550 C_3 is 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (Invitrogen). This derivative of the basic BODIPY structure contains two phenyl rings off the No. 5 and 7 carbon atoms and a propionic acid group on the No. 3 carbon atom. The carboxylate group may be used to attach the fluorophore to amine-containing molecules via a carbodiimide reaction to create an amide bond. The substituents on this BODIPY fluorophore result in alterations to its spectral properties, pushing its excitation and emission maximums up to higher wavelengths.



BODIPY 530/550 C3
4,4-Difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid
MW 416
Excitation = 535 nm
Emission = 552 nm
ε at 535 nm = 62,000 $M^{-1}cm^{-1}$

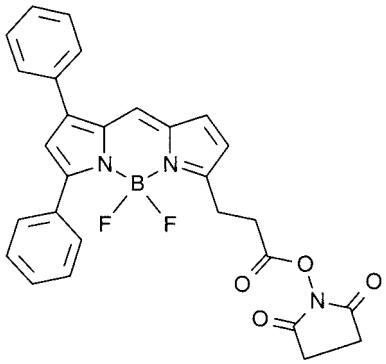
The excitation maximum for the molecule occurs at 535 nm and its emission at 552 nm. Its Stoke's shift is slightly greater than some of the other BODIPY fluorophores, producing a 17 nm separation between excitation and emission peaks. BODIPY 530/550 C_3 has an extinction coefficient in methanol of about 62,000 $M^{-1}cm^{-1}$ at 535 nm.

BODIPY 530/550 C_3 is insoluble in aqueous solution, but it may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction. Coupling to amine-containing molecules may be done using the EDC/sulfo-NHS reaction as discussed in Chapter 3, Section 1.2 (Figure 9.29). However, modification of proteins with this fluorophore probably won't yield satisfactory results, since BODIPY fluorophores are easily quenched if substitutions on a molecule exceed a 1:1 stoichiometry. For labeling molecules which contain only one amine group, such as DNA probes modified at the 5′ end to contain an amine (Chapter 27, Section 2.1), BODIPY 530/550 C_3 will give intensely fluorescent derivatives.

BODIPY 530/550 C_3-SE

BODIPY 530/550 C_3-SE is 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid, succinimidyl ester (Invitrogen). The compound is an analog of BODIPY 530/550 C_3 that

contains an active NHS ester on its propionic acid side chain (Chapter 2, Section 1.4). The ester reacts with primary amines to form stable amide bonds.



BODIPY 530/550 C_3-SE
4,4-Difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester
MW 513
Excitation = 533 nm
Emission = 550 nm
ε at 533 nm = 70,000 $M^{-1}cm^{-1}$

The excitation maximum for BODIPY 530/550 C_3-SE occurs at 533 nm and its emission at 550 nm. Its Stoke's shift is relatively small and may not be enough to avoid completely problems of excitation-light interference in emission measurements. The molecule has an extinction coefficient in methanol of about 70,000 $M^{-1}cm^{-1}$ at 533 nm.

BODIPY 530/550 C_3-SE is insoluble in aqueous solution, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction. Coupling to amine-containing molecules proceeds by nucleophilic attack at the carbonyl group, release of the NHS leaving group, and formation of an amide linkage (Figure 9.30). The reaction may be done in buffered environments having a pH range of 7–9. However, modification of proteins with this fluorophore may not yield satisfactory results, since BODIPY fluorophores are easily quenched if substitutions on a molecule result in a high molar ratio of incorporation. For labeling molecules which contain only one amine group, such as DNA probes modified at the 5′ end to contain an amine (Chapter 27, Section 2.1), BODIPY 530/550 C_3-SE will give intensely fluorescent derivatives.

Aldehyde/Ketone-Reactive BODIPY Derivatives

Hydrazide groups react with aldehyde and ketone groups to form hydrazone linkages (Chapter 2, Section 5.1). Three BODIPY derivatives are available that contain a hydrazine group modification of carboxylate side chains. Biomolecules such as proteins that don't normally possess aldehyde residues can be modified to contain them by a number of chemical means (Chapter 1, Section 4.4).

In addition, DNA and RNA may be modified with hydrazide-containing fluorophores by a transamination reaction of their cytosine residues using bisulfite as a catalyst (Chapter 27, Section 2.1) (Draper and Gold, 1980).

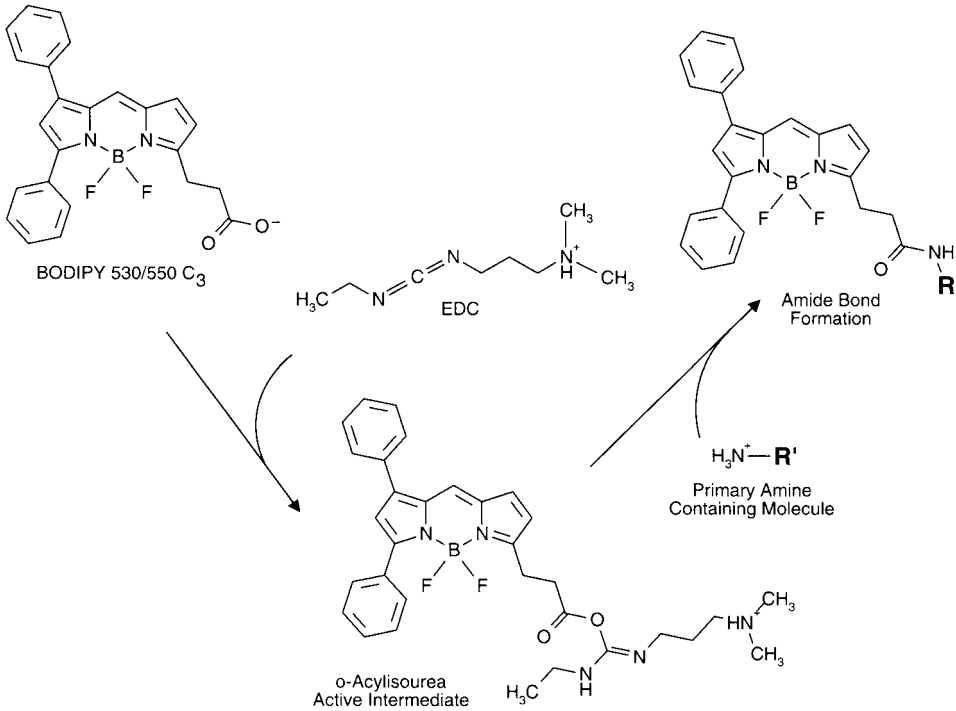


Figure 9.29 This BODIPY fluorophore contains a carboxylate group that can be attached to amine-containing molecules using a carbodiimide reaction.

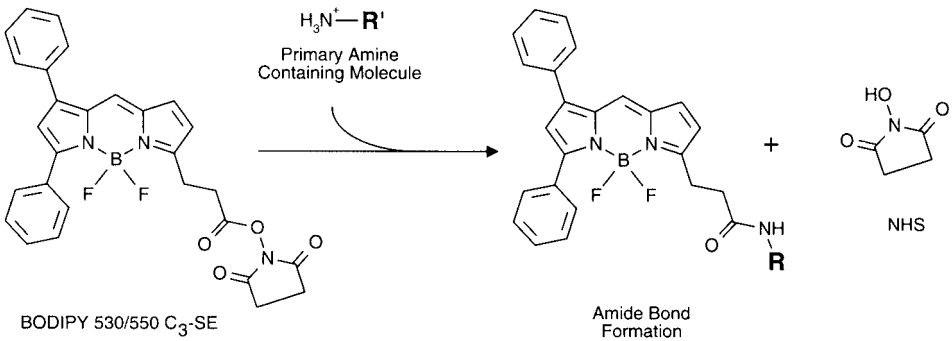


Figure 9.30 The NHS ester group of this BODIPY compound provides amine reactivity.

BODIPY 530/550 C_3 Hydrazide

BODIPY 530/550 C_3 hydrazide is 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl hydrazide, a derivative of the basic BODIPY structure that contains two phenyl rings off the No. 5 and 7 carbon atoms and a propionic acid hydrazide group on the No. 3 carbon

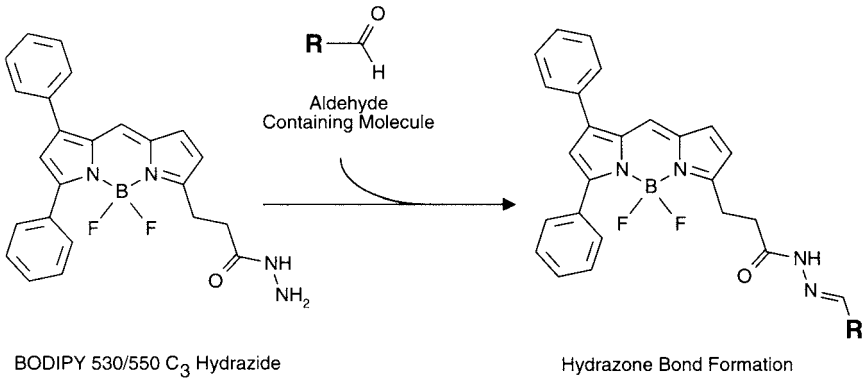
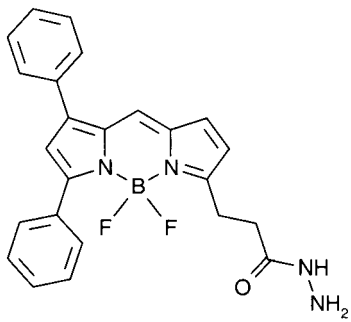


Figure 9.31 The side-chain hydrazide group of this BODIPY derivative can be used to label aldehyde-containing molecules. Glycoconjugates may be labeled after oxidation of carbohydrates with sodium periodate to produce the required aldehydes.

atom (Invitrogen). The hydrazide group reacts with aldehyde- or ketone-containing molecules to form hydrazone linkages (Figure 9.31). The compound may be used to label glycoproteins or other carbohydrate-containing molecules after oxidation of their polysaccharide portions with sodium periodate to yield aldehydes.



BODIPY 530/550 C3 Hydrazide
4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl hydrazide
MW 430
Excitation = 534 nm
Emission = 551 nm
ε at 534 nm = 79,000 $M^{-1}cm^{-1}$

The excitation maximum for BODIPY 530/550 C_3 hydrazide occurs at 534 nm and its emission at 551 nm. The molecule has an extinction coefficient in methanol of about 79,000 $M^{-1}cm^{-1}$ at 534 nm.

BODIPY 530/550 C_3 hydrazide is insoluble in aqueous solution, but may be dissolved in DMF or methanol as a concentrated stock solution prior to addition of a small aliquot to a reaction. Coupling to aldehyde-containing molecules occurs rapidly with the formation of a hydrazone linkage. The reaction may be done in buffered environments having a pH range of 5–10. However, modification of glycoproteins with this fluorophore may not yield satisfactory

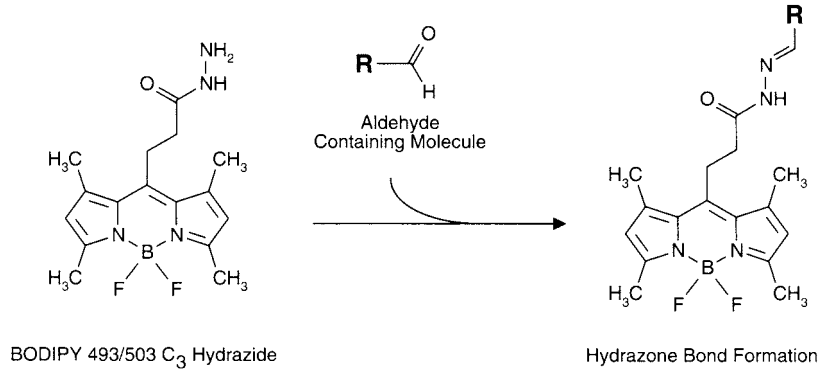
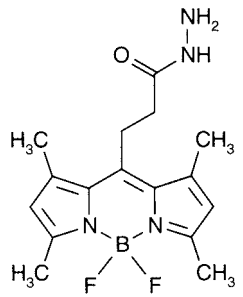


Figure 9.32 Reaction of this BODIPY fluorophore with aldehyde groups creates hydrazone linkages.

results, since BODIPY fluorophores are easily quenched if substitutions on a molecule result in a high molar ratio of incorporation.

BODIPY 493/503 C_3 Hydrazide

BODIPY 493/503 C_3 hydrazide is 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-propionyl hydrazide (Invitrogen). Unlike BODIPY 530/550 C_3 hydrazide, this BODIPY derivative contains substituents that shift to lower wavelengths the spectral characteristics of its fluorescence. The molecule is highly reactive toward aldehyde-containing compounds, including glycoproteins which have been oxidized with sodium periodate to create the requisite groups (Figure 9.32).



BODIPY 493/503 C3 Hydrazide
4,4-difluoro-1,3,5,7-tetramethyl-4-bora-
3a,4a-diaza-s-indacene-8-propionyl hydrazide
MW 334
Excitation = 498 nm
Emission = 506 nm
ε at 498 nm = 92,000 $M^{-1}cm^{-1}$

The excitation maximum for BODIPY 493/503 C_3 hydrazide occurs at 498 nm and its emission at 506 nm. Since this is an extremely small Stoke's shift, it may be difficult to avoid completely problems of excitation-light scattering interference in critical emission measurements unless sub-optimal excitation wavelengths are used. The molecule has an extinction coefficient in methanol of about 92,000 $M^{-1}cm^{-1}$ at 493 nm.

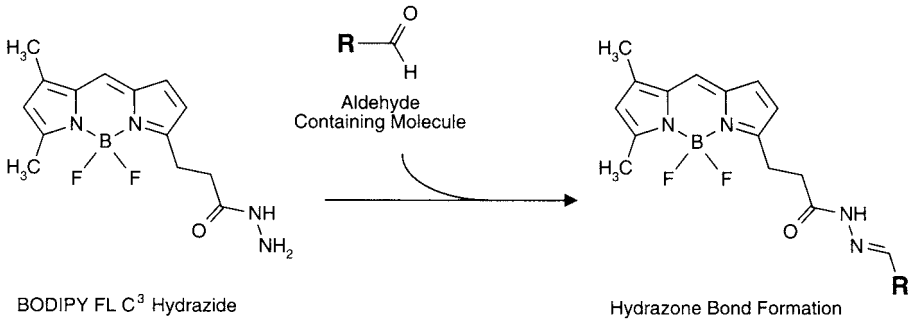
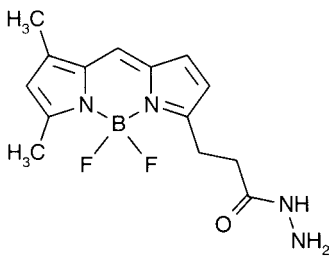


Figure 9.33 Modification of aldehyde-containing molecules can be done through this BODIPY derivative's hydrazide group.

BODIPY 493/503 C_3 hydrazide is insoluble in aqueous solution, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction mixture. Coupling to aldehyde-containing molecules occurs rapidly with the formation of a hydrazone linkage. The reaction may be done in buffered environments having a pH range of 5–10. However, modification of glycoproteins with this fluorophore may not yield satisfactory results, since BODIPY fluorophores are easily quenched if substitutions on a molecule result in a high molar ratio of incorporation. Limiting the modification level by reacting no more than a 2- to 4-fold molar excess of probe to the amount of glycoconjugate present may overcome this quenching problem.

BODIPY FL C_3 Hydrazide

BODIPY FL C_3 hydrazide is 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl hydrazide (Invitrogen). Unlike the two BODIPY hydrazide derivatives discussed above, this derivative contains substituents that produce luminescent characteristics similar to that of fluorescein, particularly with regard to fluorescing in the green region of spectrum. The molecule is highly reactive toward aldehyde-containing compounds, including glycoproteins which have been oxidized with sodium periodate to create the requisite aldehyde groups (Figure 9.33).



BODIPY FL C3 Hydrazide
4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl hydrazide
MW 306
Excitation = 503 nm
Emission = 510 nm
ε at 503 nm = 71,000 $M^{-1}cm^{-1}$

The excitation maximum for BODIPY FL C_3 hydrazide occurs at 503 nm and its emission at 510 nm. The extremely small Stoke's shift makes it difficult to avoid problems of excitation-light interference in critical emission measurements unless sub-optimal excitation wavelengths are used. The molecule has an extinction coefficient in methanol of about 71,000 $M^{-1}cm^{-1}$ at 503 nm.

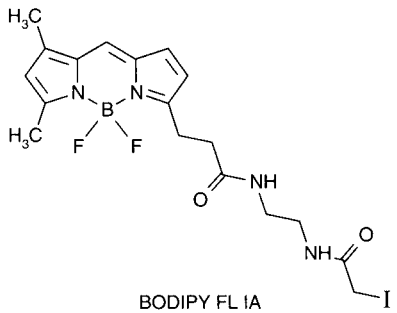
BODIPY FL C_3 hydrazide is insoluble in aqueous solution, but may be dissolved in DMF or methanol as a concentrated stock solution prior to addition of a small aliquot to a reaction mixture. Coupling to aldehyde-containing molecules occurs rapidly with the formation of a hydrazone linkage. The reaction may be done in buffered environments having a pH range of 5–10. However, modification of glycoproteins with this fluorophore may result in fluorescent quenching effects if substitutions on a molecule are at a high molar ratio of incorporation. Limiting the modification level by reacting no more than a 2- to 4-fold molar excess of probe to the amount of glycoconjugate present may help to overcome the quenching problem.

Sulfhydryl-Reactive BODIPY Derivatives

Three BODIPY derivatives are available for labeling sulfhydryl-containing molecules. The ability to label —SH groups in proteins with sulfhydryl-reactive probes provides a means of directing the modification reaction to a more limited number of sites than occurs when using amine-reactive chemistries. Directed coupling potentially can avoid active centers or binding regions. The first two sulfhydryl-reactive probes discussed in this section make use of iodoacetyl derivatives off the basic BODIPY molecule. The third probe is a bromomethyl derivative that also has good reactivity toward sulfhydryls.

BODIPY FL IA

BODIPY FL IA is *N*-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-*N*′-iodoacetylethylenediamine, an intensely fluorescent derivative of the basic BODIPY structure which is useful in modifying sulfhydryl groups (Invitrogen). The iodoacetyl group reacts with —SH groups in proteins and other molecules to form a stable thioether linkage (Figure 9.34). The reactive group is at the end of a reasonably long spacer arm, providing enough length to avoid steric problems in modifying sulfhydryls not easily accessible at the surface of macromolecules.



BODIPY FL IA
N-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)-N'-iodoacetylethylenediamine
MW 502
Excitation = 504 nm
Emission = 510 nm
ε at 504 nm = 79,000 $M^{-1}cm^{-1}$

The spectral characteristics of BODIPY FL IA somewhat mimic the green luminescence of fluorescein, thus the FL designation in its name. The excitation maximum for the probe occurs at 504 nm and its emission at 510 nm. The extremely small Stoke's shift makes it difficult to avoid problems of excitation-light interference in critical emission measurements unless sub-optimal excitation wavelengths below its excitation maximum are used. The molecule has an extinction coefficient in methanol (containing 1 percent sodium acetate and 1 percent 2-mercaptoethanol) of about 79,000 $M^{-1}cm^{-1}$ at 504 nm.

BODIPY FL IA is insoluble in aqueous solution, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction mixture. Coupling to sulfhydryl-containing molecules occurs rapidly with the formation of a thioether linkage. The reaction may be done in 50 mM sodium borate, 5 mM EDTA, pH 8.3. The main consideration is to protect the iodoacetyl derivative from light which may generate iodine and

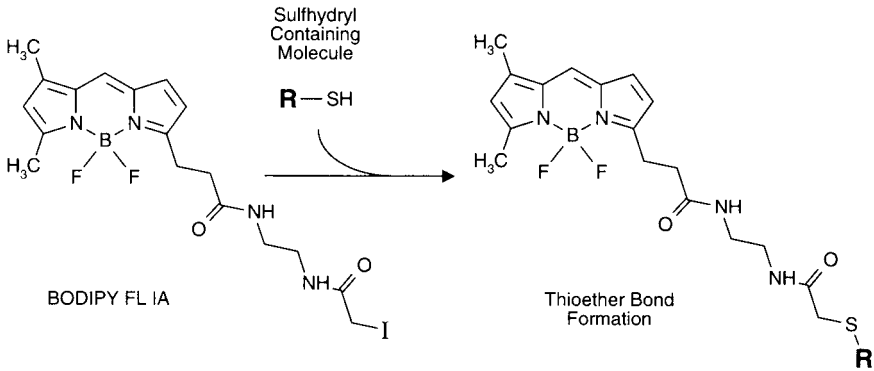


Figure 9.34 The long side chain of this BODIPY derivative contains a sulfhydryl-reactive iodoacetamide group that can couple to a thiol group to form a thioether bond.

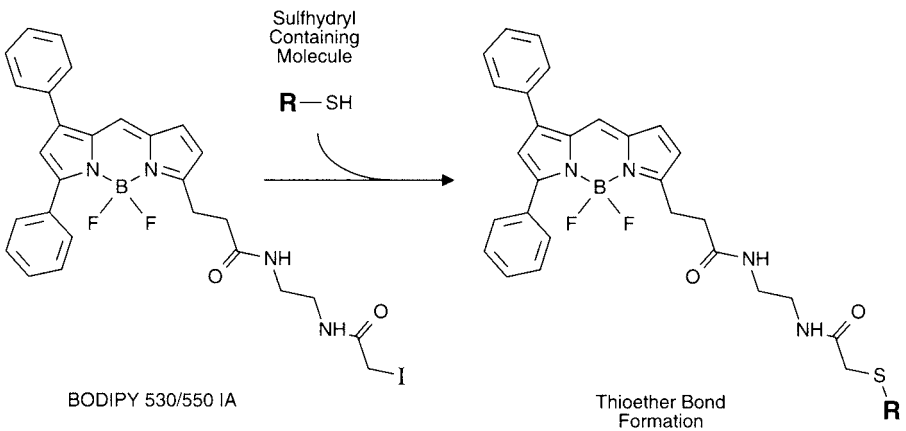
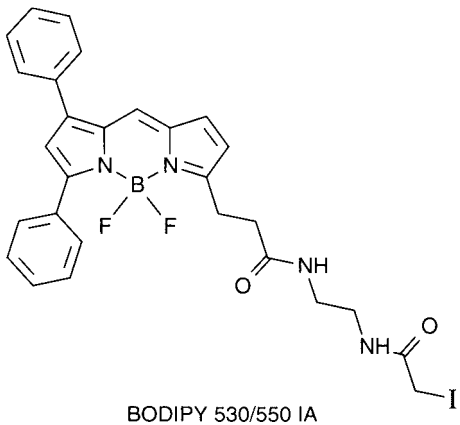


Figure 9.35 The iodoacetamide group of this BODIPY fluorophore can react with sulfhydryl-containing molecules to form thioether linkages.

reduce the reactivity of the probe. In addition, to avoid the fluorescence quenching effects that are often a problem with BODIPY probes, react no more than a 2- to 4-fold molar excess of probe to the amount of sulfhydryl groups present. Oligonucleotides containing a sulfhydryl modification at their 5′ ends (Chapter 27, Section 2.2) may be coupled with BODIPY FL IA, yielding highly fluorescent probes.

BODIPY 530/550 IA

BODIPY 530/550 IA is *N*-(4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl)-*N*′-iodoacetylethylenediamine, a derivative similar to that of BODIPY FL IA, but containing two phenyl groups rather than two methyl substituents in the No. 5 and 7 positions. This change in structure results in modulation of the spectral characteristics such that its excitation and emission wavelengths and its Stoke's shift are all increased. The spacer arm off the No. 3 carbon atom of the basic BODIPY core contains a terminal iodoacetyl group, which reacts with sulfhydryl groups in proteins and other macromolecules to create stable thioether linkages (Figure 9.35).



BODIPY 530/550 IA
N-(4,4-difluoro-5,7-diphenyl-4-bora-
3a,4a-diaza-s-indacene-3-propionyl)-
N'-iodoacetylethylenediamine
MW 626
Excitation = 534 nm
Emission = 552 nm
ε at 534 nm = 69,000 $M^{-1}cm^{-1}$

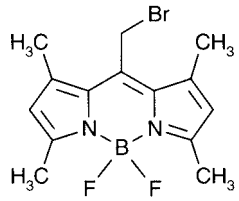
The excitation maximum for BODIPY 530/550 IA occurs at 534 nm and its emission at 552 nm. The Stoke's shift is greater than the "FL" BODIPY derivatives, having an 18 nm differential between excitation and emission wavelengths. The molecule has an extinction coefficient in methanol (containing 1 percent sodium acetate and 1 percent 2-mercaptoethanol) of about 69,000 $M^{-1}cm^{-1}$ at 534 nm.

BODIPY 530/550 IA is insoluble in aqueous solution, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small aliquot to a reaction mixture. Coupling to sulfhydryl-containing molecules occurs rapidly with the formation of a thioether linkage. The reaction may be done in 50 mM sodium borate, 5 mM EDTA, pH 8.3.

The main consideration is to protect the iodoacetyl derivative from light which may generate iodine and reduce the reactivity of the probe. To limit the degree of fluorescent quenching in the resultant conjugate, the probe should be reacted at no more than a 2- to 4-fold molar excess over the amount of target molecule present.

Br-BODIPY 493/503

Br-BODIPY 493/503 is 8-bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-3-indacene, a small BODIPY derivative containing a short, sulfhydryl-reactive bromomethyl group. The modifications to the core molecule result in modulation of its spectral characteristics such that, after conjugation, its excitation and emission wavelengths are reduced somewhat from other BODIPY probes. The reagent can be coupled to —SH containing molecules to produce a thioether linkage (Figure 9.36).



Br-BODIPY 493/503
8-Bromomethyl-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-3-indacene
MW 341
Excitation = 515 nm
Emission = 525 nm
ε at 515 nm = 55,000 $M^{-1}cm^{-1}$

The excitation maximum for Br-BODIPY 493/503 is 515 nm and its emission occurs at 525 nm when dissolved in methanol. Upon coupling to a sulfhydryl compound, however, the excitation wavelength of the adduct decreases to 493 nm and its emission drops to 503 nm. The very small 10 nm Stoke's shift may be a problem, particularly in avoiding interference due to of excitation-light scattering in critical emission measurements. Sub-optimal excitation wavelengths

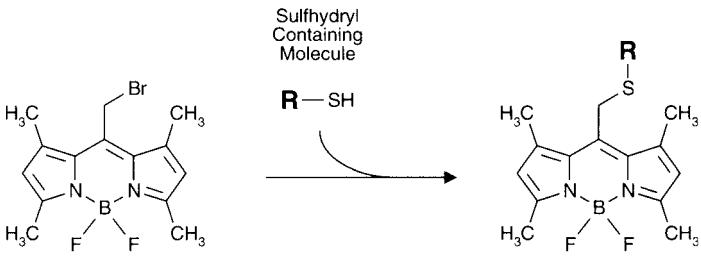


Figure 9.36 Br-BODIPY can be used to modify sulfhydryl-containing molecules to form thioether linkages.

below the excitation maximum may be used to reduce extraneous light contamination. The molecule has an extinction coefficient in methanol of about 55,000 $M^{-1}cm^{-1}$ at 515 nm.

Br-BODIPY 493/503 is insoluble in aqueous reaction mixtures, but may be dissolved in DMF or DMSO as a concentrated stock solution prior to addition of a small amount to a buffered solution. Coupling to sulfhydryl-containing molecules is rapid, leading to the formation of a thioether linkage. The reaction may be done in 50 mM sodium borate, 5 mM EDTA, pH 8.3. An important consideration is to protect the iodoacetyl derivative from light which may generate iodine and reduce the reactivity of the probe.

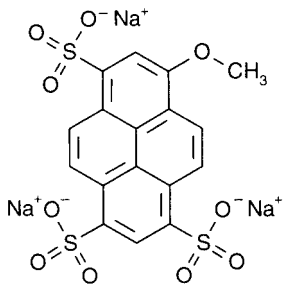
5. Cascade Blue Derivatives

Cascade Blue derivatives are fluorescent probes having strong luminescence in the blue region of the spectrum (Invitrogen). The basic Cascade Blue molecule is derived from a trisulfonated pyrene backbone (Figure 9.37) (Whitaker *et al.*, 1991). It is a fixable analog of 8-methoxypyrene-1,3,6-trisulfonic acid, which is a blue fluorescent neural tracer. The fluorophore emits light in a region removed form the luminescent signal of fluorescein or Lucifer Yellow, making it a good choice for multi-labeling applications. The dye can be used along with Lucifer Yellow CH and sulforhodamine 101 for three-color mapping of neuronal components and processes. Cascade Blue derivatives have relatively high absorptivity, good QY (typically about 0.54), excellent water solubility due to the presence of the negatively charged sulfonate groups, and good photostability. Labeling proteins and other macromolecules with Cascade Blue derivatives can be done with little fluorescent quenching due to dye–dye interactions.

The following sections discuss the most important Cascade Blue derivatives that are available for covalent modification purposes.

Amine-Reactive: Cascade Blue Acetyl Azide

One Cascade Blue derivative is available for creating linkages with amine-containing molecules. The acetyl azide functionality of this reagent reacts with primary amines at ambient temperatures or below to create amide bond derivatives (Lanier and Recktenwald, 1991; Oparka *et al.*,



8-Methoxypyrene-1,3,6-trisulfonic acid, trisodium salt

Figure 9.37 The basic structure of Cascade Blue fluorophores.

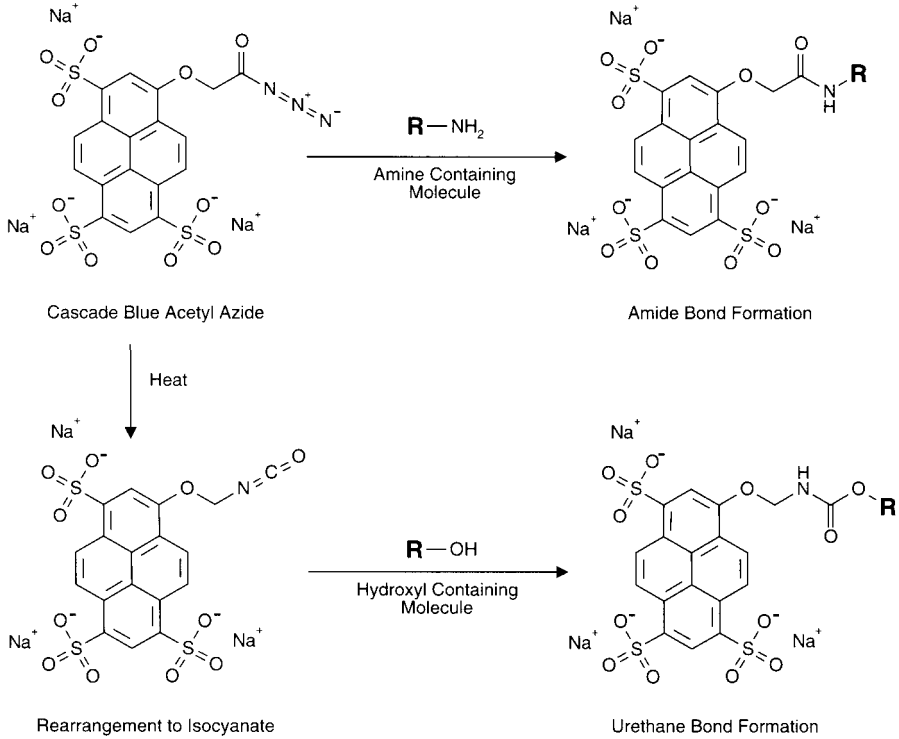
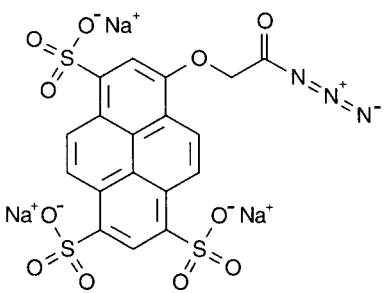


Figure 9.38 The acetyl azide group of this Cascade Blue derivative has dual functions. It can react with amine groups to form amide bonds, or it can be converted to an isocyanate at high temperatures to couple with hydroxyl functional groups, creating a carbamate linkage.

1991). At elevated temperatures (80°C in DMF), the acetyl azide group rearranges to form an isocyanate that can react with hydroxyl-containing molecules to form a urethane linkage (Figure 9.38). The Cascade Blue urethane derivatives of macromolecules are extremely fluorescent and can be detected down to femtogram quantities (Takadate *et al.*, 1985).



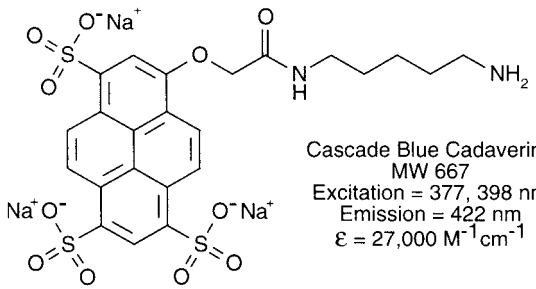
Cascade Blue Acetyl Azide
MW 607
Excitation = 375, 400 nm
Emission = 410 nm
ε at 375 nm = 27,000 $M^{-1}cm^{-1}$

This fluorophore has excitation maxima at 375 and 400 nm and an emission maximum at 410 nm. The small Stoke's shift may create some difficulty in discrete excitation without contaminating the emission measurement with scattered or overlapping light. The extinction coefficient of the molecule in water is about 27,000 $M^{-1}cm^{-1}$. Cascade Blue and Lucifer Yellow derivatives can be simultaneously excited by light of less than 400 nm, resulting in two-color detection at 410 and 530 nm.

Cascade Blue acetyl azide is soluble in aqueous solution, but the reactive azide group will hydrolyze and should be used immediately in a conjugation reaction. A concentrated stock solution may be prepared in water, dissolved quickly, and an aliquot quickly added to a buffered reaction medium. For aqueous reactions, a pH range of 7–9 is optimal. Avoid amine-containing buffers.

Carboxylate-Reactive: Cascade Blue Cadaverine and Cascade Blue Ethylenediamine

Cascade Blue cadaverine and Cascade Blue ethylenediamine both contain a carboxamide-linked diamine spacer off the 8-methoxy group of the pyrene trisulfonic acid backbone. The cadaverine version contains a 5-carbon spacer, while the ethylenediamine compound has only a 2-carbon arm. Both can be coupled to carboxylic acid-containing molecules using a carbodiimide reaction (Chapter 3, Section 1). Since Cascade Blue derivatives are water-soluble, the carbodiimide EDC can be used to couple these fluorophores to proteins and other carboxylate-containing molecules in aqueous solutions at a pH range of 4.5–7.5. The reaction forms amide bond linkages (Figure 9.39).



These fluorophores have excitation maxima at 377–378 nm and at 398–399 nm and emission maxima at 422–423 nm. The extinction coefficients of the molecules in water are about 27,000 $M^{-1}cm^{-1}$. The Cascade Blue derivatives can be used along with Lucifer Yellow

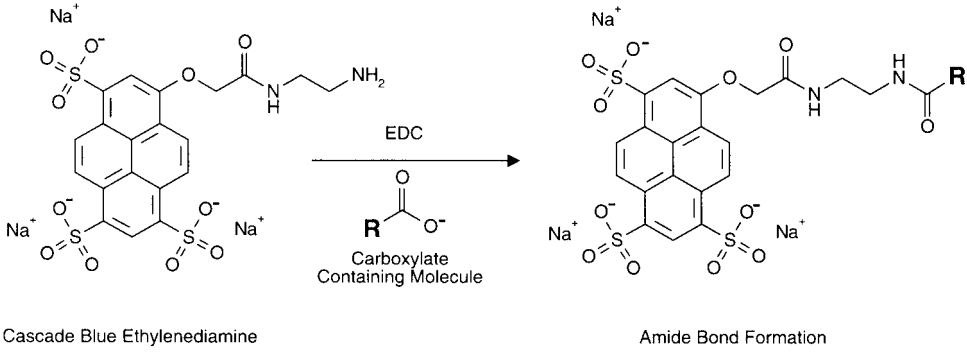


Figure 9.39 The side-chain primary amine group of this Cascade Blue derivative can be coupled to carboxylate-containing molecules using a carbodiimide reaction.

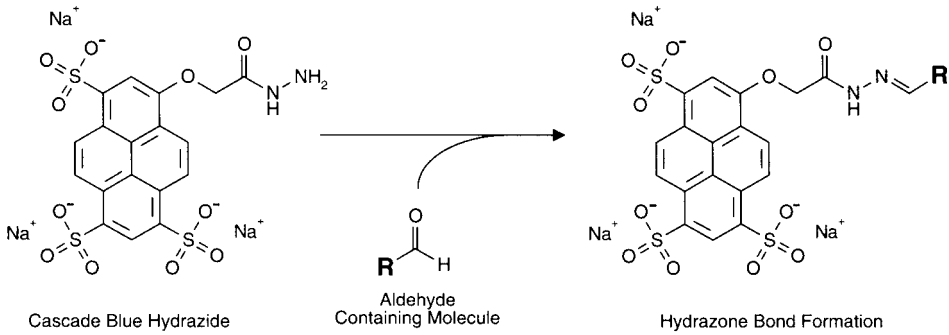


Figure 9.40 Cascade Blue hydrazide can be used to modify aldehyde-containing molecules to form hydrazone bonds.

derivatives and simultaneously excited by light of less than 400 nm, resulting in two-color detection at 422 and 530 nm.

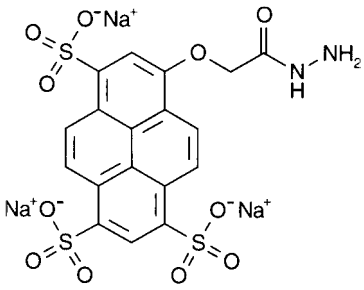
Cascade Blue diamine derivatives are soluble in aqueous solution. A concentrated stock solution may be prepared in water, dissolved quickly, and an aliquot immediately added to a buffered reaction medium. For aqueous reactions, 0.1 M MES, pH 4.7–6.5, may be used to stabilize the pH during the coupling process. Avoid amine- or carboxylate-containing buffers such as Tris or glycine, since these can compete with the coupling reaction.

Aldehyde/Ketone-Reactive: Cascade Blue Hydrazide

Cascade Blue hydrazide is a carboxy-hydrazine derivative of the 8-methoxy group on the pyrene trisulfonic acid fluorophore. Hydrazide groups react with aldehyde and ketone groups to form relatively stable hydrazone linkages (Chapter 2, Section 5.1) (Figure 9.40). Although most biomolecules don't contain aldehyde or ketone groups in their native state, carbohydrates, glycoproteins, RNA, and other molecules that contain sugar residues can be oxidized with

sodium periodate to produce reactive formyl groups. The use of modification reagents which generate aldehydes upon coupling to a molecule also can be used to produce hydrazide-reactive sites (Chapter 1, Section 4.4).

In addition, DNA and RNA may be modified with hydrazide-reactive probes by reacting their cytosine residues with bisulfite to form reactive sulfone intermediates (Chapter 27, Section 2.1). These derivatives can undergo transamination reactions with hydrazide- or amine-containing probes to yield covalent bonds (Draper and Gold, 1980).



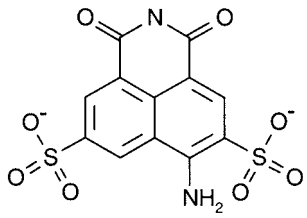
Cascade Blue Hydrazide
MW 645
Excitation = 400 nm
Emission = 420 nm
ε at 400 nm = 31,000 $M^{-1}cm^{-1}$

This fluorophore has an excitation maximum at 400 nm and an emission maximum at 420 nm. The extinction coefficient of the molecule in aqueous solution at pH 7 is about 31,000 $M^{-1}cm^{-1}$. Cascade Blue hydrazide and Lucifer Yellow derivatives can be excited simultaneously by light of less than 400 nm, resulting in two-color detection at 420 and 530 nm.

Cascade Blue hydrazide is soluble in aqueous solution, and it should be stable for awhile if protected from light. A concentrated stock solution of the reagent may be prepared in water and an aliquot added to a buffered reaction medium to facilitate the transfer of small quantities. For aqueous reactions, a pH range of 5–9 will result in efficient hydrazone formation.

6. Lucifer Yellow Derivatives

Lucifer Yellow derivatives are used extensively for cytochemical staining applications, especially in neurophysiology (Stewart, 1981a, b). The fluorophores are 3,6-disulfonate 4-aminonaphthalimide derivatives that can be further modified at their imide nitrogen to contain reactive groups suitable for conjugation with biomolecules (Figure 9.41). Cell staining with membrane-impermeant Lucifer Yellow dyes is usually done by osmotic shock, microinjection, or pinocytosis (Swanson *et al.*, 1987). Derivatives containing amines or hydrazide groups are fixable with formaldehyde or glutaraldehyde, coupling to nearby proteins or other amine-containing molecules intracellularly during the reaction. After periodate oxidation, glycoconjugates on cell surfaces or in solution may be labeled with the hydrazide derivative (Stewart, 1978). Sulfhydryl-containing molecules may be tagged with the iodoacetamide derivative.



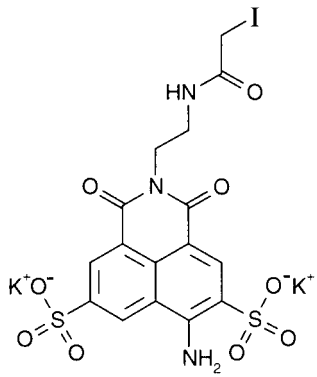
3,6-Disulfonate-4-amino-
naphthalimide

Figure 9.41 The basic structure of Lucifer Yellow fluorophores.

Lucifer Yellow probes are water-soluble to at least 1.5%. The absorbance maximum of the derivatives occurs about 426–428 nm with an emission peak at about 530–535 nm, in the yellow region of the spectrum. The QY of Lucifer dyes is about 0.25. The good intensity of luminosity from these dyes makes possible detection of small quantities of labeled molecules intracellularly. The fluorescent conjugates are readily visible in living cells at concentrations that are nontoxic to cell viability. The low molecular weight and water solubility of these dyes allow passage of labeled compounds from one cell to another, potentially revealing molecular relationships between cells.

Sulfhydryl Reactive: Lucifer Yellow Iodoacetamide

One Lucifer Yellow derivative is available for labeling sulfhydryl-containing molecules. Lucifer Yellow iodoacetamide is a 4-ethyliodoacetamide derivative of the basic disulfonate aminonaphthalimide fluorophore structure (Invitrogen). The iodoacetyl groups react with —SH groups in proteins and other molecules to form stable thioether linkages (Figure 9.42).



Lucifer Yellow Iodoacetamide,
dipotassium salt
MW 649
Excitation = 426 nm
Emission = 530 nm
ε at 426 nm = 13,000 $M^{-1}cm^{-1}$

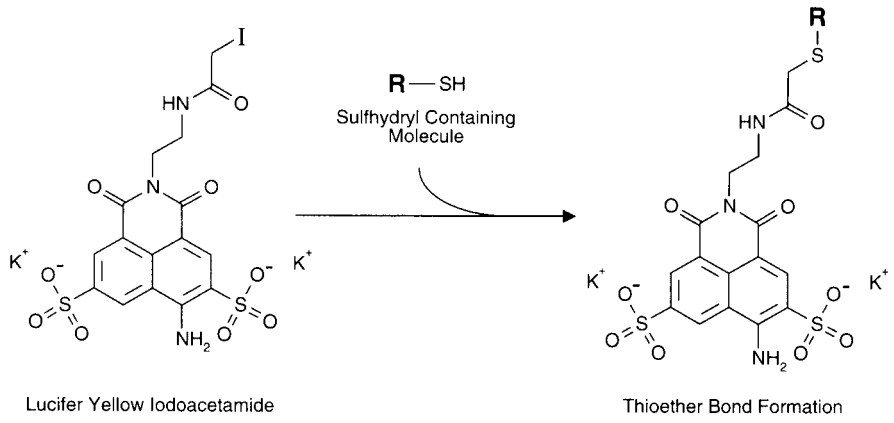


Figure 9.42 Lucifer Yellow iodoacetamide can be used to label sulfhydryl-containing molecules, forming thioether bonds.

The spectral characteristics of Lucifer Yellow iodoacetamide produce luminescence at somewhat higher wavelengths than the green luminescence of fluorescein, thus the yellow designation in its name. The excitation maximum for the probe occurs at 426 nm and its emission at 530 nm. The rather large Stoke's shift makes sensitive measurements of emission intensity possible without interference by scattered excitation light. The 2-mercaptoethanol derivative of the fluorophore has an extinction coefficient at pH 7 of about 13,000 $M^{-1}cm^{-1}$ at 426 nm.

Lucifer Yellow iodoacetamide is soluble in aqueous solution due to its negatively charged sulfonate groups. A concentrated stock solution may be prepared in water prior to addition of a small aliquot to a reaction mixture. Coupling to sulfhydryl-containing molecules occurs rapidly with the formation of a thioether linkage. The reaction may be done in 50 mM sodium borate, 5 mM EDTA, pH 8.3. The main consideration is to protect the iodoacetyl derivative from light, which will generate iodine and reduce the reactivity of the probe. The reaction may be limited to sulfhydryls (avoiding any amine derivatization) by reacting a low molar excess of probe to the amount of sulfhydryl groups present. In addition, oligonucleotides containing a sulfhydryl modification at their 5′ ends (Chapter 27, Section 2.2) may be coupled with Lucifer Yellow iodoacetamide, yielding highly fluorescent, yellow probes.

Aldehyde/Ketone Reactive: Lucifer Yellow CH

Lucifer Yellow CH is a carbohydrazide derivative of the basic disulfonate aminonaphthalimide fluorophore structure (Invitrogen). Hydrazide groups react with aldehyde and ketone groups to form relatively stable hydrazone linkages (Chapter 2, Section 5.1) (Figure 9.43). Although most biomolecules don't contain aldehyde or ketone groups in their native state, carbohydrates, glycoproteins, RNA, and other molecules that contain sugar residues can be oxidized with sodium periodate to produce reactive formyl groups. The use of modification reagents which generate aldehydes upon coupling to a molecule also can be used to produce hydrazide-reactive sites (Chapter 1, Section 4.4). In addition, DNA and RNA may be modified with hydrazide-reactive probes by reacting their cytosine residues with bisulfite to form reactive sulfone intermediates

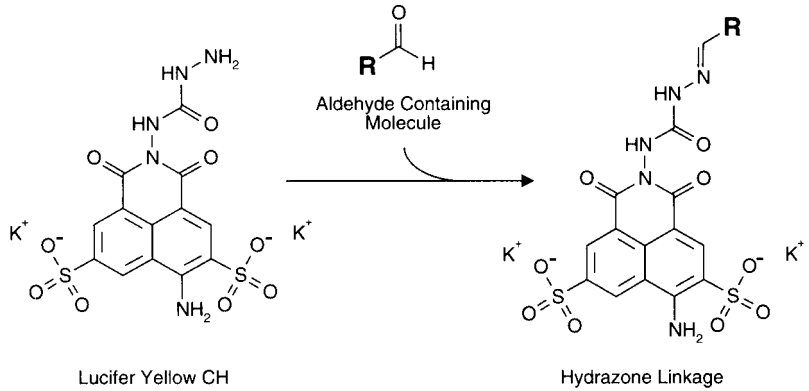
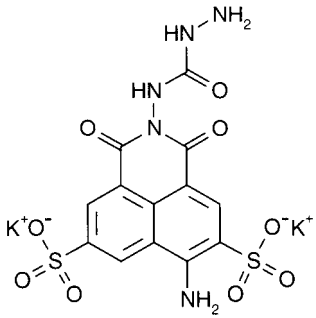


Figure 9.43 The hydrazide group of this Lucifer Yellow derivative can react with aldehyde-containing molecules to form hydrazone bonds.

(Chapter 27, Section 2.1). These derivatives can undergo transamination reactions with hydrazide- or amine-containing probes to yield covalent bonds (Draper and Gold, 1980).



Lucifer Yellow CH
MW 522 (potassium salt)
MW 457 (lithium salt)
MW 479 (ammonium salt)
Excitation = 428 nm
Emission = 533-535 nm
ε at 428 nm = 12,000 $M^{-1}cm^{-1}$

Lucifer Yellow CH is commonly used as a neuronal tracer by staining cells and then fixing them with formaldehyde or glutaraldehyde. It also can be used to label glycoproteins or glycolipids on cell surfaces after periodate oxidation (Spiegel *et al.*, 1983; Lee and Fortes, 1985). The labeling of oxidized ribonucleotides and gangliosides can be done similarly (Spiegel *et al.*, 1985; Sun *et al.*, 1988).

This fluorophore has an excitation maximum at 428 nm and an emission maximum at 534 nm. The extinction coefficient of the molecule in aqueous solution is about 12,000 $M^{-1}cm^{-1}$. Cascade Blue hydrazide and Lucifer Yellow CH derivatives can be excited simultaneously by light of less than 400 nm, resulting in the possibility for two-color detection at 420 and 534 nm.

Lucifer Yellow CH is soluble in aqueous solution, and it should be stable for awhile if protected from light. The reagent is available as three different salts of the sulfonate groups. The ammonium salt of the fluorophore is soluble to a level of 9 percent in water, while the lithium and potassium salts have a solubility of 5 and 1 percent, respectively. A concentrated stock solution of the fluorophore may be prepared in water and an aliquot added to a buffered reaction medium to facilitate the transfer of small quantities. For aqueous reactions, a pH range of 5–9 will result in efficient hydrazone formation with aldehyde or ketone residues.

7. Phycobiliprotein Derivatives

Phycobiliproteins are intensely fluorescent proteins that function as components in the photosynthetic apparatus of eukaryotic, blue-green algae and cyanobacteria (Glazer, 1981). The proteins are found as aggregates in phycobilisome particles near the chlorophyll regions (Kronick, 1986). In the native state, phycobiliproteins do not fluoresce; rather excitation energy is designed to be efficiently transferred to chlorophyll molecules for utilization in synthetic processes within the cell. Once purified, however, excitation energy is released from phycobiliproteins as strong luminosity. The fluorescent quantum efficiencies of these proteins can be as high as 0.98, far better than most synthetic probes (Grabowski and Gantt, 1978). In addition, each biliprotein contains multiple chromophoric bilin prosthetic groups, conferring extremely high absorbance coefficients to each protein molecule. B-phycoerythrin, for example, typically contains 34 chromophoric groups giving an effective, combined extinction coefficient at 545 nm of $2.4 \times 10^6\,M^{-1}\,cm^{-1}$ (Glazer and Hixson, 1977). The strong absorption bands are in the visible region of the spectrum, extending from the green to the far red wavelengths. These absorption spectra extend over a broad range of potential excitation wavelengths, allowing for versatility in the excitation source employed and creating large Stoke's shifts, thus minimizing interference from Rayleigh-scattered light (Loken *et al*., 1987).

Due to the presence of multiple fluorescent groups in each phycobiliprotein, conjugates of these molecules form extraordinarily luminescent probes. Labeling of macromolecules with phycobiliprotein derivatives can provide absorption coefficients 30-fold higher than labeling with small, synthetic fluorophores. Their ability to be monitored by fluorescing in the red region of the spectrum decreases potential interferences from indigenous biological fluorescence. The protected bilin (tetra-pyrrole) prosthetic groups are not easily affected by their external environment. They are not readily quenched by conjugation to another molecule or affected by other components in solution. The prosthetic group orientation within the protein molecules enables fluorescence to take place independent of pH or ionic strength. The excellent solubility of phycobiliproteins in aqueous solution allows easy chemical manipulation for modification or conjugation reactions, and their hydrophilic nature provides low nonspecific binding character in fluorescent detection applications.

There are three main classes of phycobiliproteins, differing in their protein structure, bilin content, and fluorescent properties. These are phycoerythrin, phycocyanin, and allophycocyanin (APC). There are two main forms of phycoerythrin proteins commonly in use: B-phycoerythrin isolated from *Porphyridium cruentum* and R-phycoerythrin from *Gastroclonium coulteri*. There also are three main forms of pigments found in these proteins: phycoerythrobilin, phycourobilin, and phycocyanobilin (Glazer, 1985). The relative content of these pigments in the phycobiliproteins determines their spectral properties. All of them,

Table 9.1 Properties of the Phycobiliproteins

Property	B-phycoerythrin	R-phycoerythrin	C-phycocyanin	Allophycocyanin
Source	*Porphyridium cruentum*	*Gastroclonium coulteri*	*Anabaena variabilis*	*Anabaena variabilis*
Subunit structure	$(\alpha\beta)_6\gamma$	$(\alpha\beta)_6\gamma$	$(\alpha\beta)_2$	$(\alpha\beta)_3$
Molecular weight	240,000	240,000	72,000	110,000
Pigment content (bilin groups)	34	34	4	6
Absorbance maximum	546 nm	566 nm	614 nm	650 nm
Molar extinction coefficient	2.4×10^6	2.0×10^6	5.8×10^5	7.0×10^5
Emission maximum	575 nm	574 nm	643 nm	660 nm

Table 9.2 Spectral Properties of Phycobiliproteins

Phycobiliprotein	Molecular weight	Absorption maximum (nm)	EC (cm^{-1} M^{-1})	Emission maximum (nm)	Fluorescence QY
B-phycoerythrin	240,000	546, 565	2,410,000	575	0.98
R-phycoerythrin	240,000	496, 546, 565	1,960,000	578	0.82
Allophycocyanin	104,000	650	700,000	660	0.68

EC = extinction coefficient; QY = quantum yield

however, have extremely high absorption coefficients ranging from a magnitude of 10^5–10^6, and excellent QY ranging from 0.51 up to 0.98.

The spectral properties of four major phycobiliproteins used as fluorescent labels can be found in Tables 9.1 and 9.2. The bilin content of these proteins ranges from a low of four prosthetic groups in C-phycocyanin to the 34 groups of B- and R-phycoerythrin. Phycoerythrin derivatives, therefore, can be used to create the most intensely fluorescent probes possible using these proteins. The fluorescent yield of the most luminescent phycobiliprotein molecule is equivalent to about 30 fluoresceins or 100 rhodamine molecules. Streptavidin–phycoerythrin conjugates, for example, have been used to detect as little as 100 biotinylated antibodies bound to receptor proteins per cell (Zola *et al.*, 1990).

Conjugation of phycobiliproteins to targeting components such as antibodies, avidin, biotin, or other molecules preserves the binding or activity of the attached constituent and does not alter the spectral characteristics of the bilin prosthetic groups. Common heterobifunctional crosslinking agents can be used to create phycobiliprotein conjugates, including SPDP (Chapter 5, Section 1.1), SMCC (Chapter 5, Section 1.3), and SMPB (Chapter 5, Section 1.6) (Oi *et al.*, 1982). These crosslinkers react with amine groups on the phycobiliproteins, producing activated intermediates able to couple with sulfhydryl-containing molecules. Thiolating reagents such as 2-iminothiolane, *N*-Succinimidyl-*S*-acetylthioacetate (SATA), and SAMSA (Chapter 1, Section 4.1) can be used to create thiols on the secondary molecule to effect the final coupling reaction.

Glazer and Stryer (1983) report on the preparation and use of tandem phycobiliprotein conjugates wherein B-phycoerythrin is crosslinked to APC to create an energy donor–acceptor pair. The B-phycoerythrin component can be excited at 545 nm, emitting energy at 575 nm that can be accepted by APC, which in turn emits light at 660 nm. The result is a large shift in the spectral characteristics from that of the individual proteins, increasing the effective Stoke's shift to

Table 9.3 Tandem Dye Fluorescence Properties

	Excitation (nm)		
Emission (nm)	488 Argon-ion laser (488)	568 Krypton-argon laser (568)	647/650 Krypton-argon (647) or red diode laser (650)
519	DyLight 488 or Alexa 488		
575	R-phycoerythrin (RPE)	R-phycoerythrin (RPE)	
590		Lissamine rhodamine	
615–630	RPE-Texas Red, RPE-Alexa 610, or RPE-DyLight 594	RPE-Texas Red, or RPE-DyLight 594	
660–665	RPE-Alexa 647 or RPE-DyLight 649	RPE-DyLight 649	DyLight 649, Allophycocyanin (APC)
702–709	RPE-Alexa-680 or RPE-DyLight 680	RPE-Alexa 680 or RPE-DyLight 680	APC-Alexa 680 or APC-DyLight 680
719–735	RPE-Alexa 700	RPE-Alexa 700	APC-Alexa 700
770–780	RPE-Alexa 750 or RPE-DyLight 750	RPE-Alexa 750 or RPE-DyLight 750	APC-Alexa 750 or APC-DyLight 750

over 100 nm. Conjugation of such tandem pairs to other proteins can create superior fluorescent reagents.

Phycobiliproteins also can be modified with organic fluorescent probes to produce tandem dyes having modulated emission properties. In this sense, dyes having excitation wavelengths that overlap with the emission wavelength of a phycobiliprotein can be used to extend the emission of the tandem complex farther into the red region. For instance, a Cy5-type dye (see Section 8, this chapter) can be used to modify R-phycoerythrin (RPE) to produce a conjugate that can be excited at the normal wavelengths used for RPE, but emit light at the Cy5 range of 660–665 nm instead of the typical 575 nm emission for the phycobiliprotein itself. Fluorescence resonance energy transfer (FRET) of excitation energy from the bilin units in RPE to the Cy5 dyes modifying the protein results in the red-shifted emission characteristics of the tandem dye construct. In an optimized tandem dye conjugate, the fluorescence emission of the phycobiliprotein is almost entirely eliminated by the FRET signaling to the organic dye.

Preparation of a series of phycobiliprotein tandem dyes allows multiplexed analysis of different targets in a sample. In addition, since RPE can be excited by the argon-ion laser at 488 nm, a fluorescein-labeled probe can be used concurrently with RPE alone and RPE-tandem conjugates to create a multiplexed system of different fluorescent probes that can be used simultaneously. Table 9.3 shows the different combinations of dyes that can be used in this type of assay with RPE and APC.

The following protocol is a generalized method for creating sulfhydryl-reactive phycobiliprotein reagents for coupling to —SH containing molecules. The procedure uses the heterobifunctional crosslinker, SPDP (Chapter 5, Section 1.1). Other amine- and sulfhydryl-reactive crosslinking agents may be used in a similar manner.

Protocol

1. Dialyze the phycobiliprotein into 50 mM sodium borate, 0.3 M NaCl, pH 8.5 (*Note*: Commercial preparations of these proteins come as an ammonium sulfate suspension).

After dialysis, adjust the protein solution to a concentration of 1 mg/ml. Higher protein concentrations may be used, but the amount of crosslinking reagent added to each ml of the reaction should be proportionally scaled up, as well. Protect the protein solution from undue exposure to light.

2. Dissolve SPDP at a concentration of 6.2 mg/ml in DMSO (makes a 20 mM stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 mM solution). If the water soluble sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to adding an aliquot to the reaction. In this case, prepare a 10 mM solution of sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of phycobiliprotein will be modified, the solid may be added directly to the reaction mixture without preparing a stock solution in water to allow accurate weighing of sulfo-LC-SPDP.
3. Add 25 µl of the stock solution of either SPDP or LC-SPDP in DMSO to each ml of the protein solution. If sulfo-LC-SPDP is used, add 50 µl of the stock solution in water to each ml of protein solution.
4. Mix and react for at least 30 minutes at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified protein from reaction by-products by dialysis using a membrane with a low molecular weight cutoff or gel filtration using desalting resin and a buffer consisting of 50 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2.

The SPDP-activated phycobiliprotein may be reacted with a sulfhydryl-containing protein to create a fluorescent conjugate linked through disulfide bonds.

8. Cyanine Dye Derivatives

One of the most popular fluorescent dye types for labeling biomolecules is built from two cationic nitrogenous ring structures linked by a polymethine bridge. One of the rings must have a quaternized nitrogen atom possessing a positive charge. The ring structure types can be highly varied, from 5- or 6-membered heterocycles to multiple fused ring systems. The polymethine bridge to a great extent determines the fluorescence character of the dye as well as contributing to the naming convention that distinguishes each dye within a family of similar structures. Thus, the general structure of all cyanine dyes can be represented by

$$X—(CH{=}CH)_n—CH{=}Y$$

where X and Y are the nitrogenous rings at both ends of the polymethine bridge and n can vary from 0 to 3. The name of a cyanine dye often has a number following it, which reflects the number of carbon atoms in the polymethine chain. Thus, if $n = 1$, the dye is called a tricyanine or Cy3, if $n = 2$ it is a pentacyanine or Cy5, and if $n = 3$ it is a heptacyanine or Cy7.

The longer the polymethine bridge in a cyanine dye, the higher the absorbance and emission wavelengths become. In general, for each incremental increase of n, the absorbance and

emission characteristics of the dye increase by about 100 nm. Thus, Cy3 dyes typically display excitation and fluorescence in the mid-500 nm range, Cy5 has spectral properties in the mid-600 nm range, and Cy7 in the mid-700 nm range.

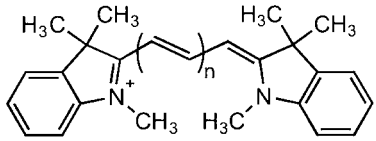
Though the bridge structure affects significantly the spectral character of a cyanine dye, the heterocyclic rings also can contribute heavily to its properties. For instance, the structure of the ring systems can highly affect the absorptivity and brightness of a particular cyanine dye. Some heterocycle structures, such as the commonly used indol rings of many commercial cyanine compounds, have extremely high extinction coefficients and thus provide intensely bright fluorescent labels. Other constituents on the heterocyclic rings can provide a blue shift or a red shift in the spectral characteristics of a dye. This is the basis for creating intermediate Cy dyes having fluorescent properties between the standard ones. For instance, Cy5.5 falls between the emission wavelengths of Cy5 and Cy7, completely due to an alternative fused ring structure (e.g., benzo-indolium groups, see Figure 9.44). The relative fluorescence effects of different ring systems and numerous possible substitutions on these rings often can be predicted from decades of investigation and data (Gruber, 2002). Thus, many commercial suppliers of these dyes have fine-tuned their properties to make them more suitable for certain applications.

The nonreactive base structures of cyanine dyes (or carbocyanines) have been used for many years as components in photographic emulsions to increase the range and sensitivity of film and also in CD-R and DVD-R optical disks to record digital information. A major innovation came when Ernst *et al.* (1989) and Waggoner *et al.* (1993) recognized that cyanine dyes would make excellent labels for fluorescence detection, and for this reason, they synthesized reactive dye derivatives, which then could be covalently attached to proteins and other molecules.

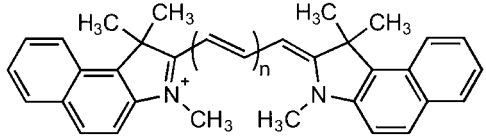
The nitrogenous ring structures at each end of a cyanine dye can be the same (symmetrical structure) or different (unsymmetrical), depending on the heterocycle type and constituents on the rings. Reactive cyanine dyes nearly always contain unsymmetrical structures due to the presence of a reactive arm on one end to facilitate conjugation to biomolecules or the presence of one or more negatively charged groups, which are designed to increase water solubility.

The earliest cyanine dye labels were relatively hydrophobic due to the lack of hydrophilic groups or having only a minimal number of charged groups on the molecules. These dyes, while intensely fluorescent, often cause aggregation or precipitation of labeled proteins, especially if more than just a few fluorescent molecules are attached to a single biomolecule. Dye–dye interactions due to ring stacking or hydrophobic interactions also cause fluorescence quenching, because energy transfer can take place between dye molecules, negating the emission of light.

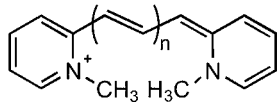
To make cyanine dyes more biocompatible for protein labeling, sulfonate groups typically are added to the ring systems or at the ends of short spacer arms protruding from the base dye structure. The addition of sulfonate groups provides a negative charge character that helps solubilize the cyanine dye in aqueous solution and prevents dye–dye interactions through like charge repulsion. In general, the more sulfonates that a cyanine dye possesses the greater will be its hydrophilicity and the lower its tendency to bind nonspecifically to hydrophobic structures on proteins or other molecules. Cyanine dyes of this type having from two to four sulfonate groups are available commercially, with three or four sulfonates per dye giving the best results for bioconjugation purposes in aqueous solution.



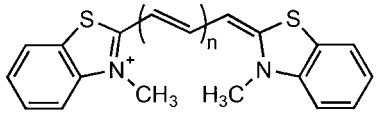
Indolium-based cyanine dye



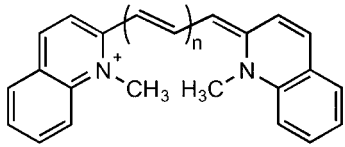
Benzo-indolium-based cyanine dye



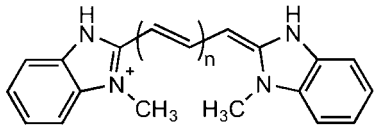
Pyridinium-based cyanine dye



Thiazolium-based cyanine dye



Quinolinium-based cyanine dye



Imidazolium-based cyanine dye

Figure 9.44 The common nitrogenous ring structures of cyanine-type dyes. Indolium-based dye derivatives are the most frequently used due to the high extinction coefficient of the indol groups.

Cyanine dyes also are used as labels for oligonucleotide probes. Unlike the hydrophilic cyanine dyes valuable for protein labeling, the use of dye-phosphoramidite compounds to synthesize DNA or RNA probes typically requires the use of more hydrophobic dye structures to make them compatible with the solvents and reactions of oligonucleotide synthesis. Thus, indol cyanines containing few or no sulfonates are used in these applications to label oligos for applications such as array detection, hybridization assays, and RT-PCR.

In addition, small unsymmetrical cyanine compounds have been designed that bind to the minor groove of DNA to measure DNA concentration or stain DNA by fluorescence. These derivatives often contain a positive charge character with short crescent shapes to wrap around the helical structure of double-stranded oligonucleotides. As minor groove binding dyes interact with DNA, they typically undergo a slight twist in their molecular structure, which dramatically increases fluorescence QY and brightness. For example, the minor groove binding dye BOXTO displays a 300-fold increase in fluorescence when binding to double-stranded DNA (Karlsson *et al.*, 2003).

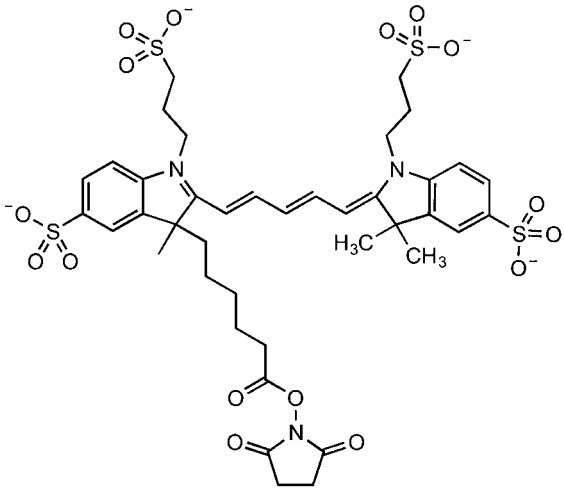
Most companies selling cyanine dyes do not reveal their exact structures. This likely is due to each company keeping proprietary the small synthetic tweaks that create unique fluorescence properties for their dyes. However, some structures are available through published documents, such as patents and early publications (Leung *et al.*, 2005). Figure 9.45 illustrates some of these structures, which may not reflect precisely what any one company actually offers today, but it gives an idea of the types of modifications that can be done to add water solubility and reactivity.

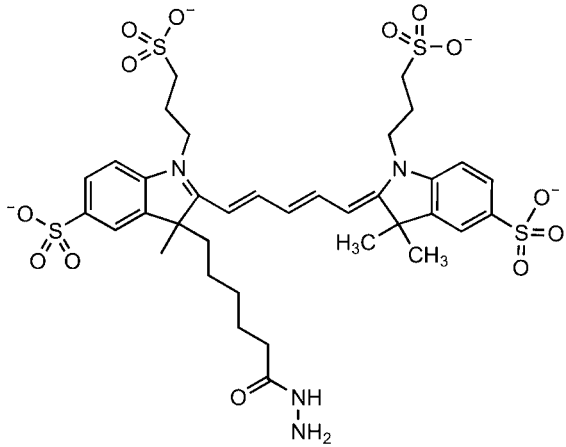
Cyanine dyes are commercially available in a number of different structural configurations containing appropriate reactive groups to couple with many of the major functional groups (Thermo Fisher, GE Healthcare, Invitrogen, Sigma, Dyomics, Atto-Tec, and Denovo Biolabels). Most of these dyes contain sulfonate groups to increase their biocompatibility, but careful comparisons should be made, as most companies do not provide structures. The following sections describe some of the reactive cyanine dye reagents and the protocols that are used to label proteins and other molecules.

Amine-reactive Cyanine Dyes

Amine-reactive cyanine dyes typically contain an NHS ester group on the end of a short hydrocarbon spacer for attachment to biomolecules. The NHS ester can react with amine groups on proteins to form amide bond linkages (Figure 9.46). This reaction is efficient at physiological pH or under slightly more alkaline conditions. While poly-sulfonated dye molecules are very water-soluble, it is best first to prepare a stock solution in organic solvent to prevent NHS ester hydrolysis prior to adding a small aliquot to a reaction mixture.

The following protocol for protein labeling with an NHS ester-cyanine dye is based on the Thermo Fisher instructions for use of DyLight dyes. When preparing a fluorescently labeled protein the degree of modification should be optimized to provide maximal fluorescence signal while not affecting the activity or binding potential of the protein for other molecules. In fact, dye loadings of greater than about 8 fluorescent labels per protein will result in fluorescence quenching due to energy transfer between dye molecules. Higher loadings will not result in greater fluorescence signals, only greater potential to interfere with protein activity. Therefore, limit the molar excess of dye over protein to a level that will provide less than about 8 substitutions per protein molecule.





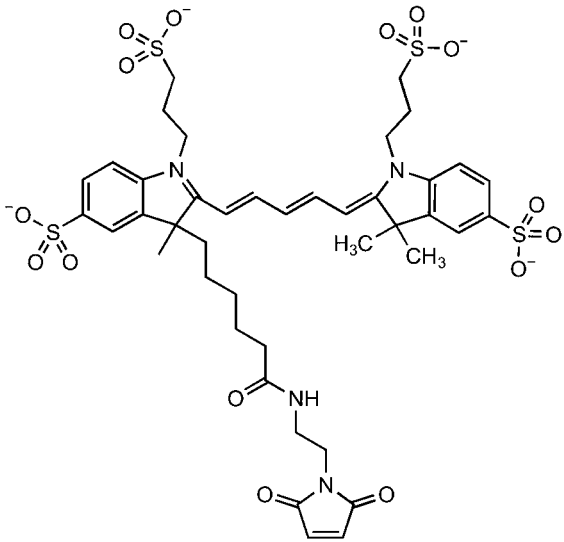


Figure 9.45 Typical Cy5 derivatives contain negatively charged sulfonate groups for water solubility and a side chain terminating in a reactive group for covalent coupling. The most common reactive groups are an NHS ester for labeling amines, a maleimide group for coupling to thiols, and a hydrazide group for labeling aldehydes.

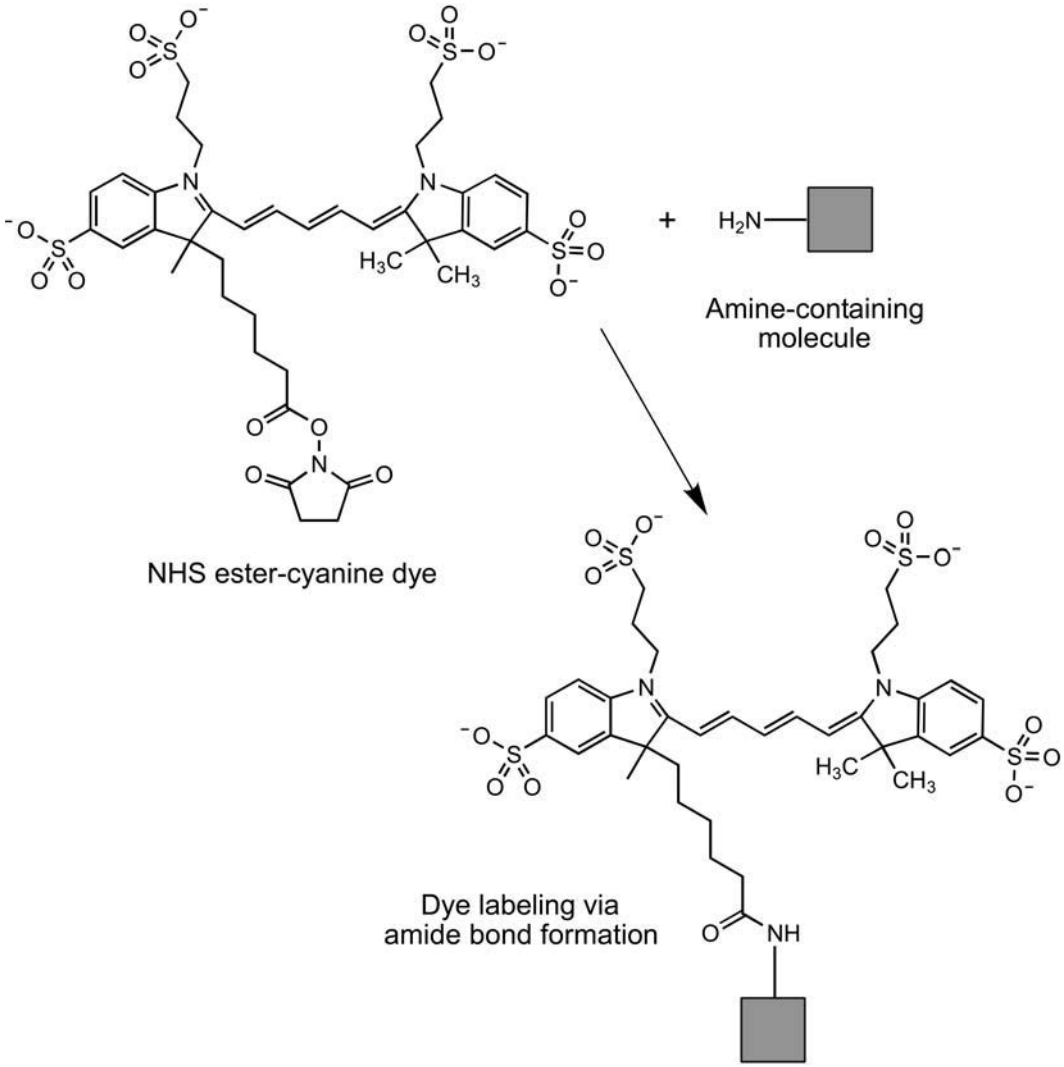


Figure 9.46 An NHS ester-containing cyanine dye can be used to label amine-containing proteins or other molecules to form amide bonds.

Protocol

1. Dissolve a protein or antibody to be labeled with an NHS ester-cyanine dye in 0.1 M sodium phosphate buffer, pH 7.2–7.5, at a concentration of 1–10 mg/ml. The higher the concentration of protein, the greater will be the yield of the labeling reaction. Other buffers also may be used, including borate buffer or carbonate buffer, but higher pH reactions will result in greater hydrolysis of the NHS ester and may decrease labeling

efficiency. Avoid amine-containing buffers such as Tris or imidazole. Also, avoid the addition of thiol reducing agents or glycerol, as these also will react with the NHS ester. If the protein concentration is below 1 mg/ml, the molar excess of dye added to the reaction will have to be greatly increased to maintain yield of the reaction.

2. In a fume hood, dissolve the NHS ester-cyanine dye in DMF at a concentration of 10 mM. Protect all dye solutions from light.
3. With mixing, add a quantity of the dye solution to the protein solution to provide the desired molar excess of dye over protein. For instance, for an antibody dissolved in buffer at 10 mg/ml, the addition of 66 µl of dye solution will give a 10-fold molar excess of dye over protein.
4. React for 1 hour at room temperature with gentle mixing.
5. Purify the labeled protein from excess dye and reaction by-products using dialysis or gel filtration on a desalting resin.

Thiol-reactive Cyanine Dyes

Cyanine dyes that have a thiol-reactive group typically are maleimide derivatives. Maleimide groups react with sulfhydryls under neutral pH conditions to form a thioether linkage (Figure 9.47). Since thiols are present in proteins in limited amounts, dye labeling through these groups often will result in modifications that occur only at discrete locations on the molecule. Labeling thiol groups also may be done using peptides that have been synthesized with terminal cysteine residues or using oligonucleotides containing a 5′ thiol group.

The pH of a maleimide conjugation reaction should be in the range of 6.5–7.5 to assure specificity toward thiol groups. Higher pH conditions will begin to result in cross-reactions with amines through a Michael addition process. Coupling to proteins can be done through disulfide reduction or through use of a thiolation reagent (Chapter 1, Section 4.1). Reduction of disulfides may be done using immobilized TCEP (Thermo Fisher), which effectively forms free thiols on proteins and peptides while not contaminating a reaction with the reducing agent.

The following protocol is based on the methods recommended by Thermo Fisher for use of the cyanine dye DyLight 649. Antibody reduction is based on the methods of Sun *et al.* (2005), which results in partially reduced bispecific immunoglobulin containing available thiols in the hinge region for labeling.

Protocol

Partial Reduction of Antibody IgG

1. Dissolve an antibody in 50 mM sodium phosphate, 150 mM NaCl, 10 mM EDTA, pH 7.2 (reaction buffer), at a concentration of 1–10 mg/ml.
2. Dissolve TCEP in reaction buffer at a concentration of 10 mM and readjust the pH if necessary.
3. Add a quantity of the TCEP solution to the antibody solution with mixing to achieve a 2.75 M excess of the reducing agent over the amount of antibody present.
4. Incubate for 2 hours at 37°C.

The reduced antibody can be used immediately to label with a maleimide-based cyanine dye using the following protocol.

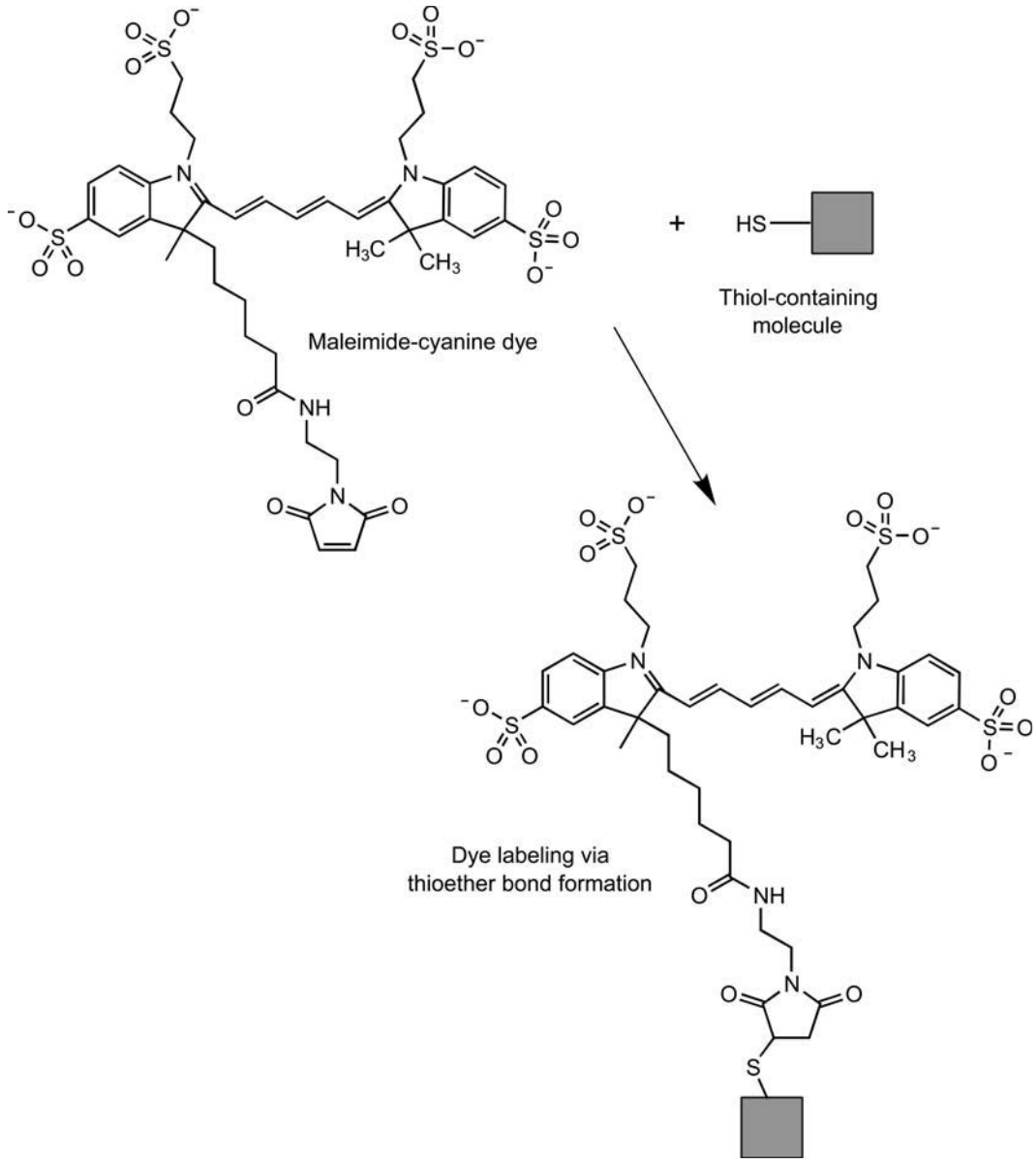


Figure 9.47 A maleimide-containing cyanine dye can be used to label thiol-containing molecules to form thioether bonds.

Labeling of Reduced Antibody with Thiol-Reactive Cyanine Dye

5. In a fume hood, dissolve the maleimide-cyanine dye in DMF at a concentration of 10 mM. Protect all dye solutions from light.
6. With mixing, add a quantity of the dye solution to the antibody solution to provide the desired molar excess of dye. For instance, for an antibody dissolved in buffer at

10 mg/ml, the addition of 66 µl of dye solution will give a 10-fold molar excess of dye over protein.
7. React for 2 hours at room temperature with gentle mixing.
8. Purify the labeled protein from excess dye and reaction by-products using dialysis or gel filtration on a desalting resin. Protect the labeled protein from light to avoid photobleaching the dye and losing fluorescence intensity.

Carbonyl-reactive Cyanine Dyes

Cyanine-type dyes containing hydrazide functional groups may be used to label molecules containing carbonyl groups, especially aldehydes (Figure 9.48). Aldehydes may be created on carbohydrates, sugars, and glycans by periodate oxidation, which cleaves the carbon–carbon bonds between diols to form reactive formyl groups. Thus, glycoproteins and other glycoconjugates can be labeled specifically with a cyanine fluorescent dye only through carbohydrate components, which often avoids binding sites or active centers. Hydrazide-containing cyanine dyes also may be used to detect glycoproteins in cells, tissues, gels, or on Western blots after periodate oxidation. In this regard, bands containing glycoproteins can be detected in a sample separately from the rest of the non-glycosylated protein pool (Thermo Fisher).

Glycan molecules that have been released from a protein by enzymatic means also can be labeled at their reducing ends by a hydrazide-containing cyanine dye. This reaction results in a single fluorescent label on the C-1 carbon of the inner most sugar of the glycan tree. The labeled glycan can be tracked through separation steps or detected for its specific interactions with carbohydrate binding proteins (see Chapter 1, Section 4.6).

Hydrazide-based cyanine dyes are reactive with common formaldehyde fixatives for cell and tissue studies. This enables these dyes to function as general stains for protein-rich areas within cells, and they get crosslinked into place by the formaldehyde reaction process.

The following protocol relates to the labeling of glycoproteins with hydrazide-cyanine dyes. Similar methods can be used to detect glycoproteins in other applications, such as within electrophoresis gels or on blots. For methods related to the labeling of glycans at their reducing ends with hydrazide reagents, see Chapter 11, Section 6.

Protocol

1. Dissolve a glycoprotein to be labeled in 50 mM sodium phosphate, pH 7 (reaction buffer), at a concentration of 1–10 mg/ml. For sialic acid modification, place the sample in ice to cool to near 0°C.
2. Dissolve sodium meta-periodate in reaction buffer at a concentration of 10 mg/ml (0.046 M). Protect from light. To obtain approximately a 1 mM concentration of sodium periodate in the reaction solution (suitable for oxidizing only sialic acid residues), add 21.8 µl of this stock solution to each ml of the glycoprotein solution to be oxidized. Maintain the solution on ice. For general oxidation of carbohydrates other than just sialic acid, add 218 µl of the stock solution to obtain an approximate final concentration of 10 mM periodate in the reaction. Use room temperature conditions for general carbohydrate oxidation. Wrap the vial containing the reaction solution with aluminum foil to protect from light. The use of an amber vial also is suitable for this purpose.

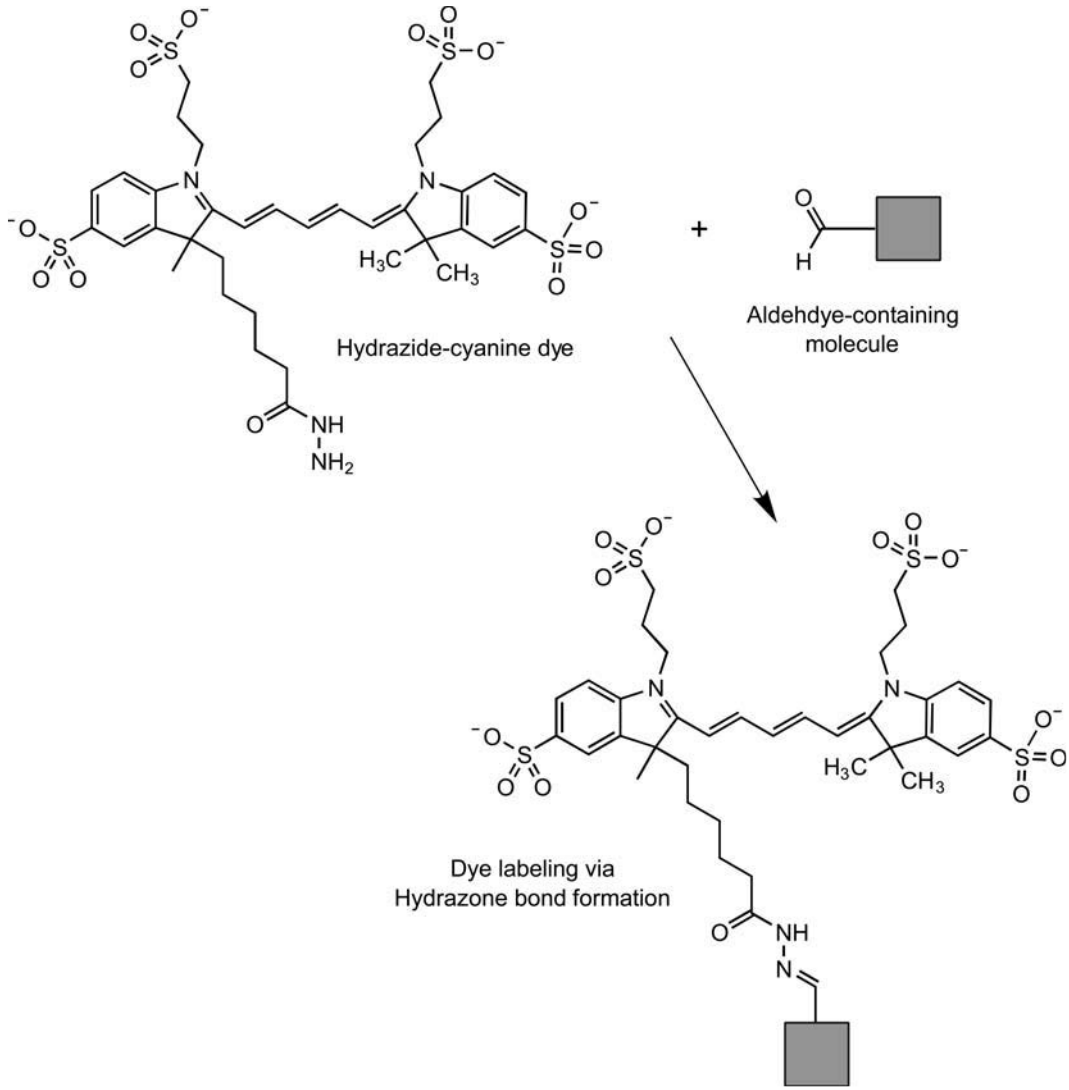


Figure 9.48 A cyanine dye containing a hydrazide group can be used to label glycans at their reducing end or other reducing sugars, forming a hydrazone linkage. Glycoproteins also can be labeled after periodate oxidation to form aldehyde groups.

3. React for 15–30 minutes at room temperature.
4. Quench the reaction by immediate gel filtration on a desalting column. If a dextran-based resin is used for the chromatography, the support itself will react with sodium periodate to quench excess reagent. Alternatively, *N*-acetylmethionine may be added to quench the reaction, because the thioether of the methionine side chain will react with periodate to form sulfoxide or sulfone products (Geoghegan and Stroh, 1992). In addition, sodium

sulfite (Na_2SO_3) was used by Stolowitz *et al.* (2001) to quench the periodate oxidation of horseradish peroxidase (HRP) in solution. To quench the reaction with cellular samples, wash the cells with buffer to remove remaining traces of periodate.

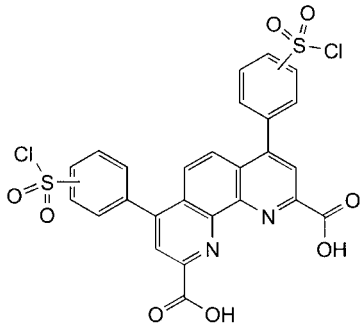
5. In a fume hood, dissolve the hydrazide-cyanine dye in DMF at a concentration of 10 mM. Protect all dye solutions from light.
6. With mixing, add a quantity of the dye solution to the oxidized antibody solution to provide the desired molar excess of dye. For instance, for an antibody dissolved in buffer at 10 mg/ml, the addition of 66 µl of dye solution will give a 10-fold molar excess of dye over protein.
7. React for 2 hours at room temperature with gentle mixing.
8. Purify the labeled protein from excess dye using dialysis or gel filtration on a desalting resin. Protect the labeled protein from light to avoid photobleaching the dye and losing fluorescence intensity.

9. Lanthanide Chelates for Time-resolved Fluorescence

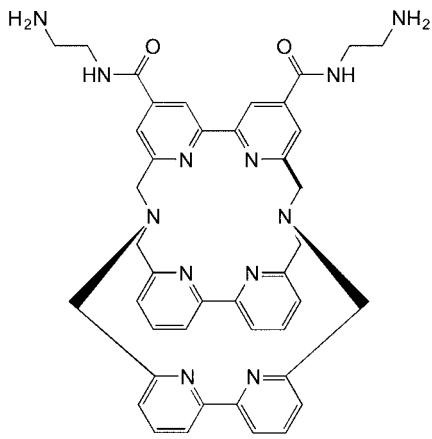
Lanthanide metals are sometimes called rare earths and are located in period 6 of the periodic table of elements with atomic numbers 57–71, beginning with lanthanum (although there is some discrepancy over exactly where they start and end). Due to their unique electronic properties, lanthanide ions display luminescent properties with very sharp emission peaks. Unfortunately, the metal ions alone have extremely low extinction coefficients of no more than about 1–10 $M^{-1}cm^{-1}$, which makes them poorly fluorescent. The dim nature of lanthanide luminescence can be overcome by associating the metal ion with a chromophore group that can act as a light antenna. Lanthanide metals can form nine coordination bonds with ligands, creating three-faced centered trigonal prism structures. Coordinated in this way in an organic-chelate structure that also contains a strongly absorbing chromophore nearby, the dimness of lanthanide metal luminescence can be transformed into an extraordinarily bright complex. The appropriate antenna group is one that can absorb energy and transfer it to the lanthanide metal, which then emits light at specific wavelengths, depending on what lanthanide element is in the coordination complex. For a review of chelating compounds for lanthanide luminescence, see Arnaud and Georges (2003). Some examples of antenna-chelate structures are shown in Figure 9.49 (Figure 9.50).

Fluorescent labels built from lanthanide-chelating groups of this type are extremely useful in biological applications due to their large Stoke's shift (nearly 290 nm), no overlap between excitation and emission peaks, and very long fluorescent lifetimes. When bound by a chelating group that completely envelops and interacts with most of the lanthanide's nine possible coordination sites, the metal also is protected from any solvent quenching effects, which may reduce luminescence (Pietraszkiewicz *et al.*, 1993). If the antenna-chelating group also contains a reactive group, then the lanthanide chelate complex can be covalently linked as a label on targeting molecules or affinity ligands. The result is one of the most intensely fluorescent probes available for biological detection applications.

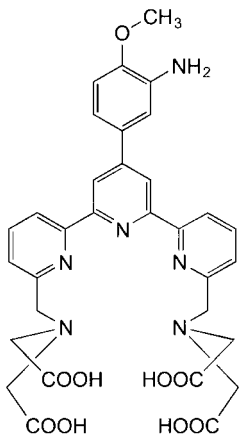
The fluorescence lifetimes of lanthanide chelates are among the longest of all fluorescent compounds. Whereas most organic fluors or inorganic nanocrystals have lifetimes measured in the nanosecond or picosecond range, lanthanide chelates typically emit light for microseconds to milliseconds. The advantage of this long-lived emission is that time-gated measurements can



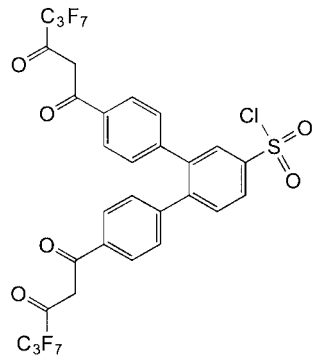
BCPDA;
4,7-Bis-(chlorosulfophenyl)-
1,10-phenanthroline-2,9-dicarboxylic acid



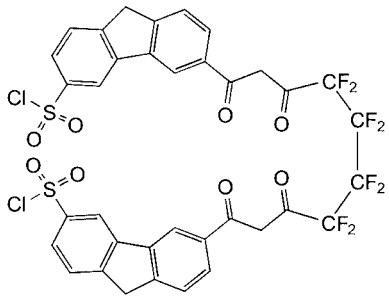
TBP;
trisbipyridine cryptate



TMT;
4'-(3-isothiocyanato-4-methoxyphenyl)-
6,6"-bis[N,N-bis(carboxymethyl)
aminomethyl)-2,2';6',2"-terpyridine



BHHCT;
4,4'-bis(1",1",1",2",2",3",3"-
heptafluoro-4",6"-hexanedion-6"-yl)
chlorosulfo-o-terphenyl



BCOT;
1,10-bis(8'-chlorosulfo-
dibenzothiophene-2'-yl)-4,4,5,5,6,6,7,7, -
octafluorodecane-1,3,8,10-tetraone

Figure 9.49 Examples of antenna-chelator compounds that have been used for lanthanide luminescence.

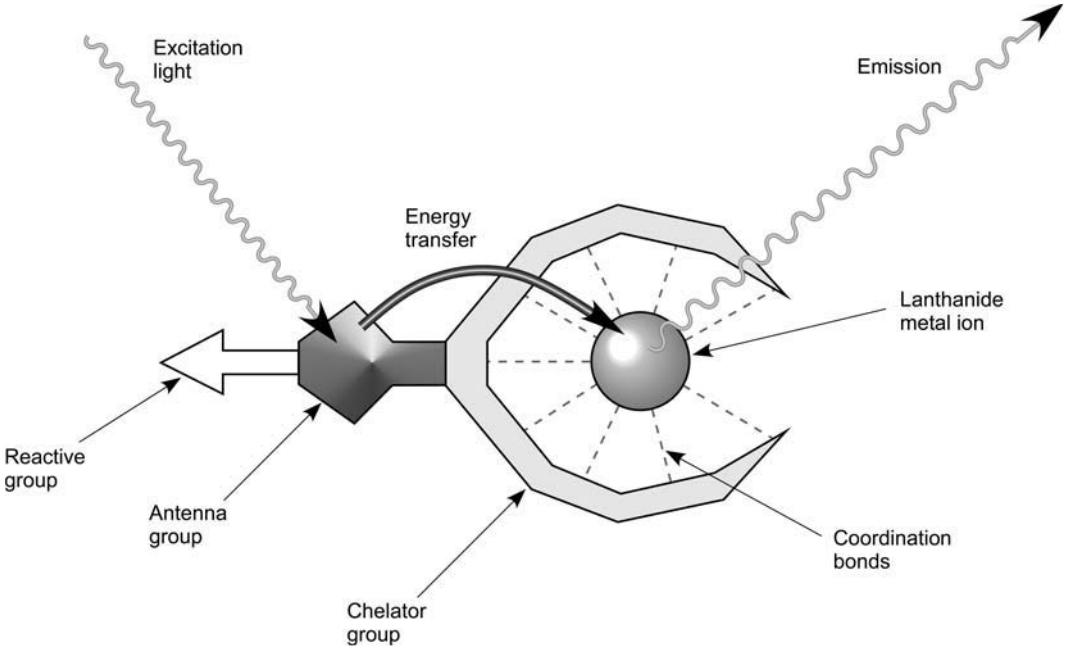


Figure 9.50 The principle of enhanced lanthanide luminescence using antenna-chelating compounds. The organic dye antenna group absorbs energy and transfers it to the lanthanide metal ion coordinated within the chelate structure. The best chelating groups fully coordinate the metal and protect it from the quenching effects of water. The lanthanide atom then emits light at a longer wavelength, which is characteristic of the lanthanide metal. Lanthanide chelates also contain a reactive group that can be used to attach covalently the label to targeting molecules.

be taken, which detect lanthanide luminescence only long after emission from other organic fluors has decayed to zero. In complex biological samples, often there are many organic components that have fluorescent properties, especially in the lower visible or UV regions of the spectrum. When using lanthanide chelates as fluorescent probes and labels, any indigenous sample fluorescence can be eliminated before the chelate emission is measured. This "time-resolved" luminescence technique virtually abolishes background fluorescence and makes detection and assays using lanthanide chelates among the most sensitive fluorescence measurements possible. For reviews of time-resolved fluorescence using lanthanide chelates in biological assays, see Soini and Kojola (1983); Soini *et al.* (1990); Diamandis and Christopoulos (1990); Hemmila (1985); Diamandis (1993); and Hemmila (1998).

Different lanthanide metals also produce different emission spectrums and different intensities of luminescence at their emission maximums. Therefore, the relative sensitivity of time-resolved fluorescence also is dependent on the particular lanthanide element complexed in the chelate. The most popular metals along with the order of brightness for lanthanide chelate fluorescence are europium(III) > terbium(III) > samarium(III) > dysprosium(III). For instance, Huhtinen *et al.* (2005) found that lanthanide chelate nanoparticles used in the detection of human prostate antigen produced relative signals for detection using europium, terbium, samarium, and dysprosium of approximately 1.0:0.67:0.16:0.01, respectively. The emission

wavelengths for each of these lanthanides also are different. Terbium has a main peak of luminescence in range of 545 nm, dysprosium at about 575 nm, europium at about 615 nm, and samarium at approximately 645 nm (although there are other emission bands for all of these lanthanide metals as well).

In assays, europium chelates usually produce the greatest sensitivity in an assay, followed closely by terbium chelates. Another advantage of europium is that it has the longest fluorescent lifetime of all the lanthanides, making it the best choice for time-resolved applications. For these reasons, most applications of lanthanide fluorescence use europium or terbium chelates to modify biological molecules, as they provide the brightest conjugates achievable when using this technology.

Another advantage of lanthanide chelates is their lack of self-quenching effects. Since the excitation and emission peaks don't overlap for a given lanthanide metal, the chelates don't quench each other if modified at high density on other molecules or immobilized close together on surfaces or particles. Therefore, creating complexes having multiple lanthanide-chelating groups is a strategy that can dramatically increase fluorescence. This property differs from organic fluorescent reagents, as dyes usually start to quench if as few as 6–8 fluorescent labels modify a single protein. By contrast, it is possible to form polymer or particle labels containing dozens, hundreds, or even thousands of lanthanide-chelating groups, which are capable of increasing the fluorescent signal in assays equal to the sum of the total number of groups present. Using this approach, Huhtinen *et al.* (2005) created nanoparticle labels containing hundreds of fluorescent lanthanides. Similarly, Scorilas *et al.* (2000) created a polyvinylamine polymer chains with multiple europium chelates and also added biotin labels to form huge complexes with streptavidin in solution. Such reagents increase dramatically the lanthanide luminescence signal in immunoassays or other detection applications beyond that possible with standard organic fluors.

Lanthanide chelates also can be used in FRET applications with other fluorescent probes and labels (Figure 9.51). In this application, the time-resolved (TR) nature of lanthanide luminescent measurements can be combined with the ability to tune the emission characteristics through energy transfer to an organic fluor (Comley, 2006). TR-FRET, as it is called, is a powerful method to develop rapid assays with low background fluorescence and high sensitivity, which can equal the detection capability of enzyme assays (Selvin, 2000).

FRET signaling is limited by the distance requirements for energy transfer between fluorescent molecules. The donor molecule must be constrained within the immediate molecular vicinity of the acceptor fluorescent molecule. FRET systems can be described in terms of the Forster radius, which is the distance at which energy transfer efficiency is 50 percent (Forster, 1948; Lakowicz, 1999). For energy transfer between organic fluors, the Forster radius is usually no more than about 15–20 Å. This means that for FRET signaling to occur with high retention of fluorescence yield, the donor and acceptor molecules must be in extreme proximity, held much closer than the radius of the average globular protein.

Lanthanide chelates have distinct advantages in FRET systems, because their Forster radius can be on the order of 80–100 Å. This means that two biological molecules coming together in solution, one labeled with a donor lanthanide chelate and the other labeled with an acceptor organic fluor, usually are positioned close enough to undergo efficient energy transfer. Thus, lanthanide TR-FRET assays can be developed that are completely homogeneous in nature; taking advantage of both the long lifetime of fluorescence and the efficient energy transfer characteristics of a long Forster radius. For instance, an antibody labeled with a europium

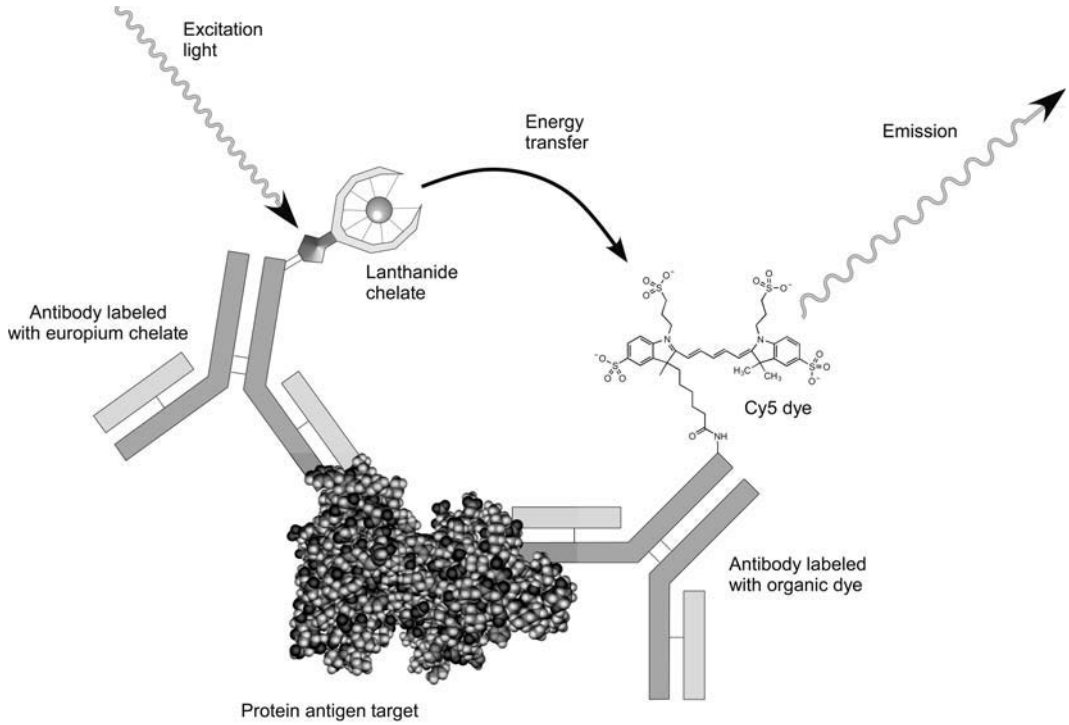


Figure 9.51 Time-resolved FRET assay systems involve energy transfer between the lanthanide chelate and an organic dye that are brought together as two labeled molecules bind to an analyte. In this illustration, an antibody labeled with a lanthanide chelate is used along with a Cy5-labeled antibody to detect a protein target in solution. Excitation of the lanthanide label results in energy transfer and excitation of the cyanine dye only if they are held within close enough proximity to allow efficient FRET to occur. Under these conditions, excitation of the lanthanide chelate results in cyanine dye emission, which will not occur if the labeled antibodies have not bound to a target.

chelate can be used along with another antibody labeled with an organic fluorescent label able to absorb energy from the lanthanide. If both antibodies recognize different epitopes on a target antigen, then a solution phase assay can be designed wherein the FRET signal is observed only if both antibodies are bound to the antigen. The excess labeled antibodies in solution still will be free to diffuse throughout the sample and thus not be held in enough proximity to undergo energy transfer. The result is low background interference, even though excess fluor is not washed away.

To make a luminescence resonance energy transfer (LRET) system work, the proper choice of acceptor fluor must be made for the lanthanide chelate. The acceptor must have an excitation band corresponding to or overlapping an emission band of the lanthanide label, so that excitation energy may be transferred. Although energy transfer is never 100 percent efficient as would be evident by a complete disappearance of the lanthanide emission band, the efficiency can be high. To maximize the yield of fluorescence transfer, the acceptor also should be an efficient absorber with a high extinction coefficient and have bright fluorescent properties (high

QY) to assure good signal in the final assay application. In this regard, one of the best acceptor molecules for lanthanide luminescence is the phycobiliprotein, APC. APC often is paired with europium chelates to create LRET assays. The protein's multiple fluorescent bilin groups enhance its ability to receive energy transfer from the luminescence of the donor europium. In an europium–APC system, excitation in the UV range with subsequent energy transfer results in emission in the red region of the spectrum, giving an effective Stoke's shift of over 300 nm. Combine the FRET signal with a time-gated measurement and the result is a highly sensitive assay with virtually no interference from other biological or organic molecule fluorescence.

Organic fluorescent dyes with the appropriate spectral properties also can be paired with lanthanide chelates in FRET systems. For instance, many rhodamine dyes and the cyanine dye Cy5 have ideal excitation wavelengths for receiving energy from a nearby europium chelate. The LeadSeeker assay system from GE Healthcare incorporates various Cy5-labeled antibodies for developing specific analyte assays. In addition, if using a terbium chelate as the donor, then a Cy3 fluorescent dye can be used in assays as the acceptor.

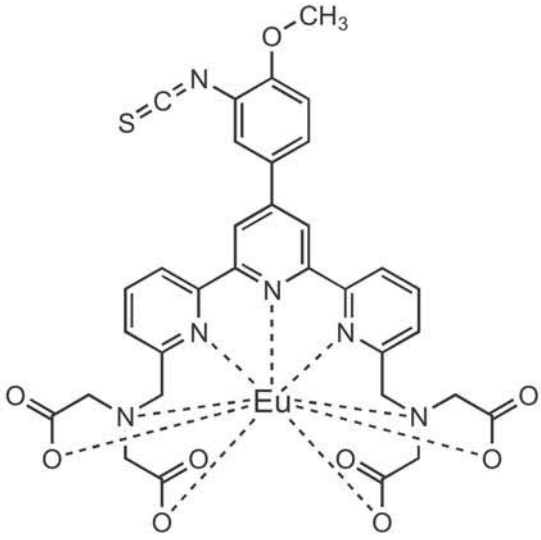
Antenna-chelate molecules for lanthanide luminescence must have a reactive group to allow conjugation with targeting molecules to be useful for TR-FRET in biological applications. Some of the reactive groups that have been designed for polyaminocarboxylate chelates include the amine-reactive isothiocyanate derivatives (Li and Selvin, 1997), thiol-reactive maleimide groups, and pyridyl disulfide groups (Chen and Selvin, 1999).

Two of the more common chelate structures are built from an EDTA or DTPA backbone (Chapter 10, Section 1). Depending on the method of antenna group addition, the DTPA-chelate structure can have a maximum of eight coordination groups consisting of carboxylates and amines to hold the lanthanide atom. An EDTA-chelating group can have up to six coordination groups per chelate molecule. The remaining coordination sites are taken up by water molecules in aqueous solution, which potentially can quench luminescence, because they are effective acceptors of excitation energy from the lanthanide. Both EDTA- and DTPA-chelating groups with antenna molecules do perform well in luminescent assays. However, it is better to have a chelating group that can coordinate with all or a majority of the nine coordination bonds on a lanthanide metal ion. This avoids the potential for quenching effects from other chelators in solution or from water itself. In this regard, reagents built from a DTPA-chelating group potentially are better than those based on EDTA.

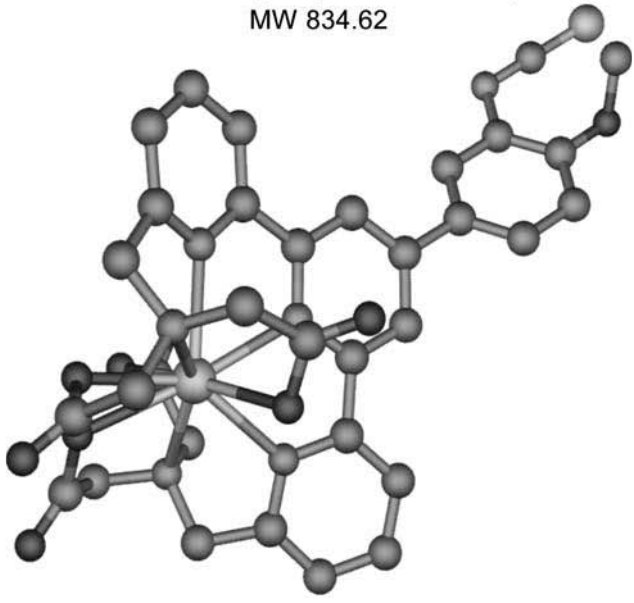
In some cases, the initial lanthanide-chelating group may not have an antenna group built into its structure. One of the early commercial applications of europium luminescence in a TR-FRET-based assay system used an isothiocyanatophenyl-EDTA or an isothiocyanatophenyl-DTPA derivative to modify proteins (Delfia system from Wallac). This system uses an isothiocyanate chelate group to carry the lanthanide and attach it to antibodies and other molecules, but it doesn't have an antenna group. In an assay, this system requires the use of a secondary fluorescence enhancer to create the lanthanide fluorescence signal. This secondary reagent typically is β-naphthoyltrifluoroacetone, which when added in excess at the end of an assay extracts the lanthanide metal from the nonfluorescing chelate and forms another soluble chelate that is highly fluorescent.

More advanced designs incorporate efficient antenna groups directly into the chelating structure of the label. For instance, the terpyridine-*bis*(methylenamine) tetraacetic acid (TMT) chelator of europium developed by Saha *et al.* (1993) has multiple pyridine rings and two iminodiacetic acid groups on each side to create a chelator having nine coordination sites. The pyridine groups provide the antenna structure and also supply nitrogen atoms with unshared pairs of

electrons to coordinate with the lanthanide metal ion. The result is a protected lanthanide that cannot be quenched easily by water or other matrix components. Complexed with europium, this label can be excited at 340 nm and provide sharp emission peaks at wavelengths of 589, 599, 618 (maximum emission), 623, 651, 689, 697, and 702 nm. The largest peaks in the >600 nm range can optimally excite Cy5 dyes, such as the hydrophilic DyLight 649 (Section 8, this chapter) and create an effective TR-FRET system for homogeneous assay design.



Terpyridine-bis (methylenamine)
tetraacetic acid (TMT) chelate of europium
MW 834.62



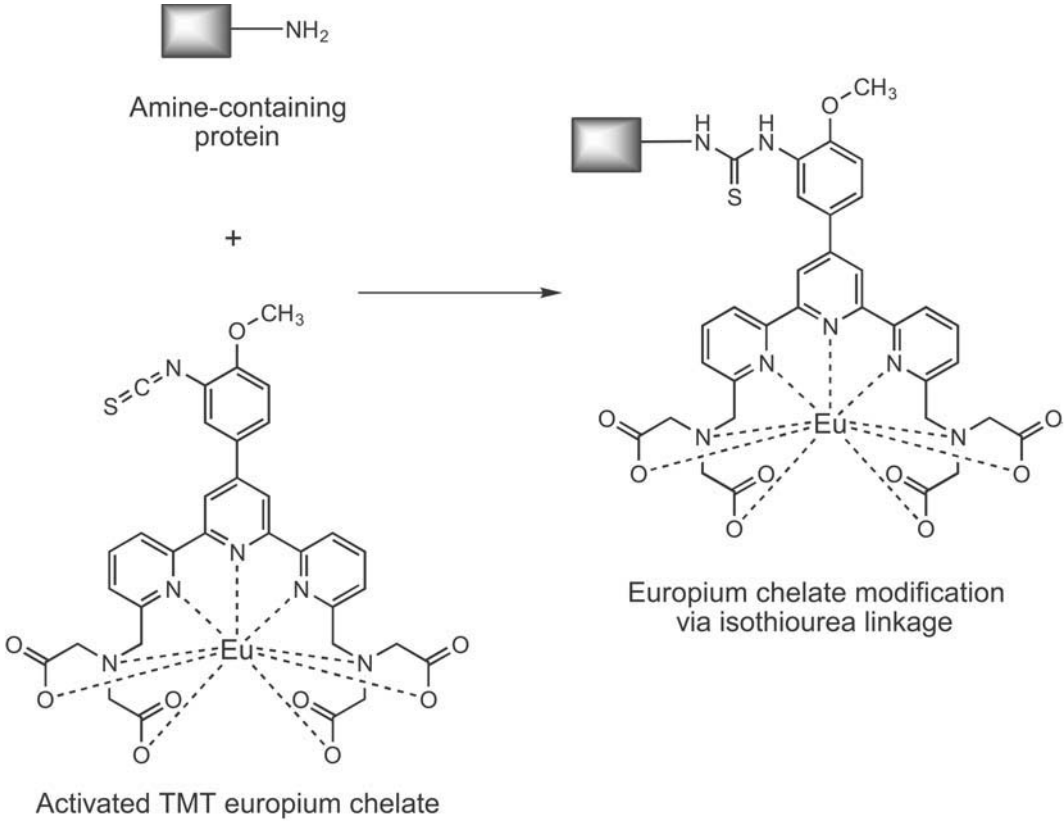


Figure 9.52 The isothiocyanate group of a TMT europium chelate can be used to label amine-containing proteins and other molecules to result in an isothiourea bond.

The TMT chelator containing an amine-reactive isothiocyanate group can be used to label antibodies, proteins, or other molecules using the following protocol (Figure 9.52).

Protocol

1. In a fume hood, dissolve the TMT chelator in a 1:1 mixture of DMF:DMSO at a concentration of 10 mg/ml.
2. Dissolve a protein to be labeled in 0.1 M sodium bicarbonate, pH 9.0, at a concentration of 1–10 mg/ml.
3. With mixing, add a quantity of the TMT chelator to the protein solution to provide a 5- to 10-fold molar excess of reagent. Optimization may have to be done to determine the best ratio of chelator to protein for the intended application.
4. React at 4°C overnight with gentle mixing.
5. Purify the labeled protein by gel filtration or dialysis.

As in the structure of the TMT chelator group, pyridine derivatives long have been known to be enhancers of lanthanide luminescence (Thomas *et al.*, 1978). One such compound,

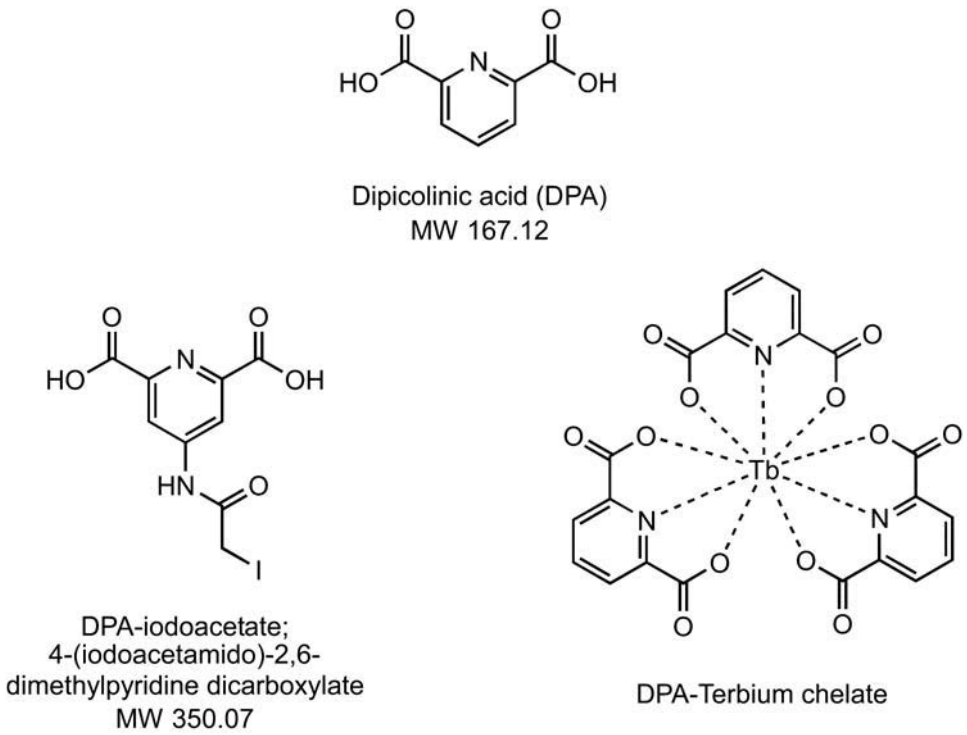


Figure 9.53 DPA derivatives have been used as potent enhancers of lanthanide luminescence. Three DPA groups can coordinate with a terbium ion. The iodoacetate derivative of DPA has been used to label covalently molecules for lanthanide luminescence.

dipicolinic acid (DPA), contains two carboxylates on both sides of a pyridine core, which provides three coordination sites per molecule to complex with lanthanides. In solution, three DPA molecules can coordinate with one lanthanide ion to fully surround the metal and protect it from quenching by water or other molecules. Each DPA pyridine group can absorb energy in the UV region and transfer it to the central lanthanide, which results in strong emission at characteristic wavelengths.

Lamture and Wensel (1995) synthesized a unique modification of the basic DPA structure to make the chelator bifunctional and thus able to react with functional groups on other molecules. This reactive DPA reagent, 4-(iodoacetamido)-2,6-dimethylpyridine dicarboxylate, contains an iodoacetyl group on the C-4 of the pyridine ring, thus making it particularly reactive with thiol groups and amines at higher pH. This derivative was used to create a large polymeric-chelating structure from poly-L-lysine that contained 50–100 DPA units along its length. Loading this complex with terbium(III) ions resulted in highly intense luminescence. The remaining amines on poly-L-lysine finally were succinylated to create carboxylates for coupling to proteins via carbodiimide conjugation (Figures 9.53 and 9.54).

Another antenna group that is particularly effective for lanthanide luminescence is carbostyril derivatives (7-amino-4-methyl-2(1H)-quinolinone), which can be attached to many chelating

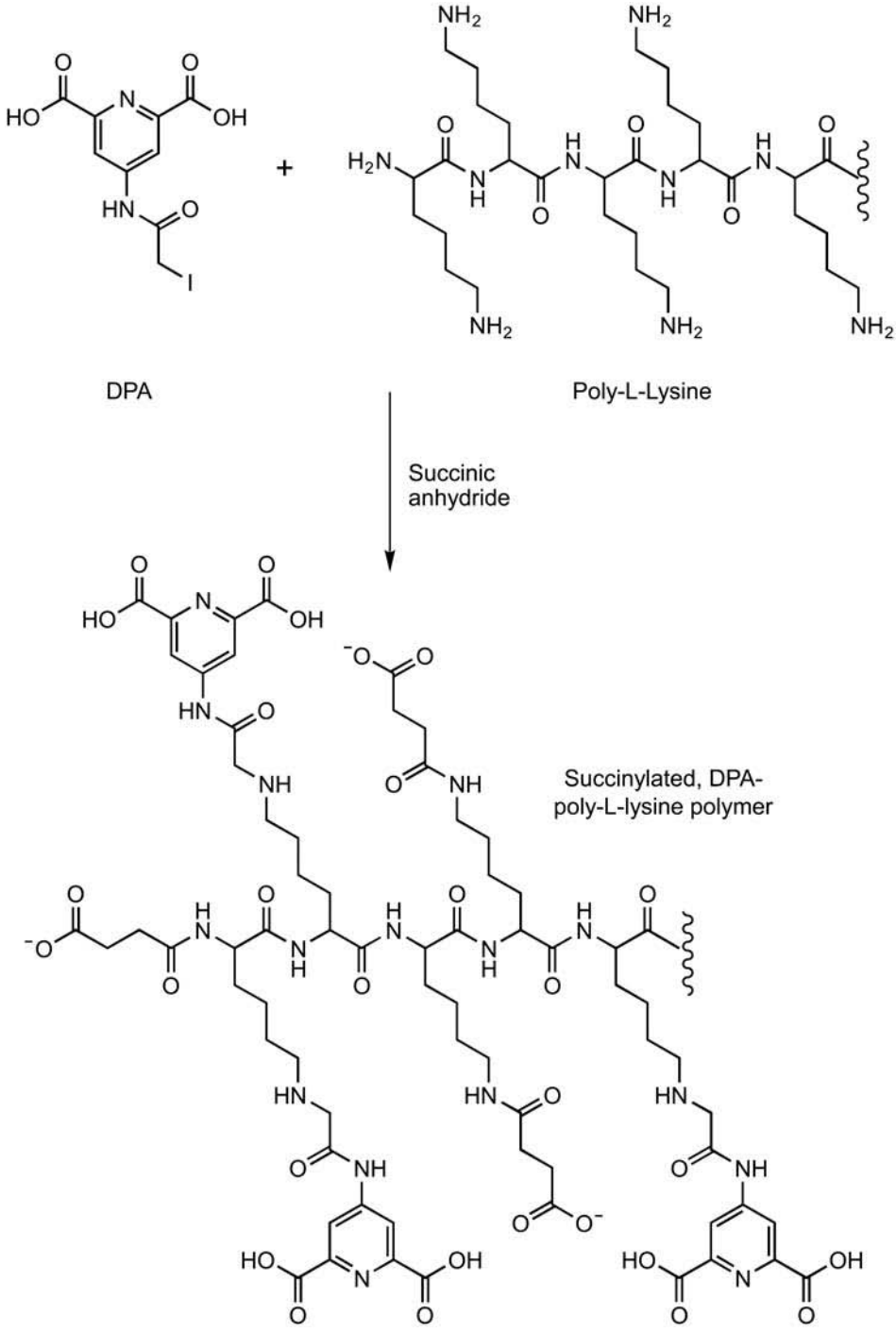


Figure 9.54 The iodoacetamide derivative of DPA has been used to create a chelating polymer of lanthanide metals using poly-L-lysine as the backbone.

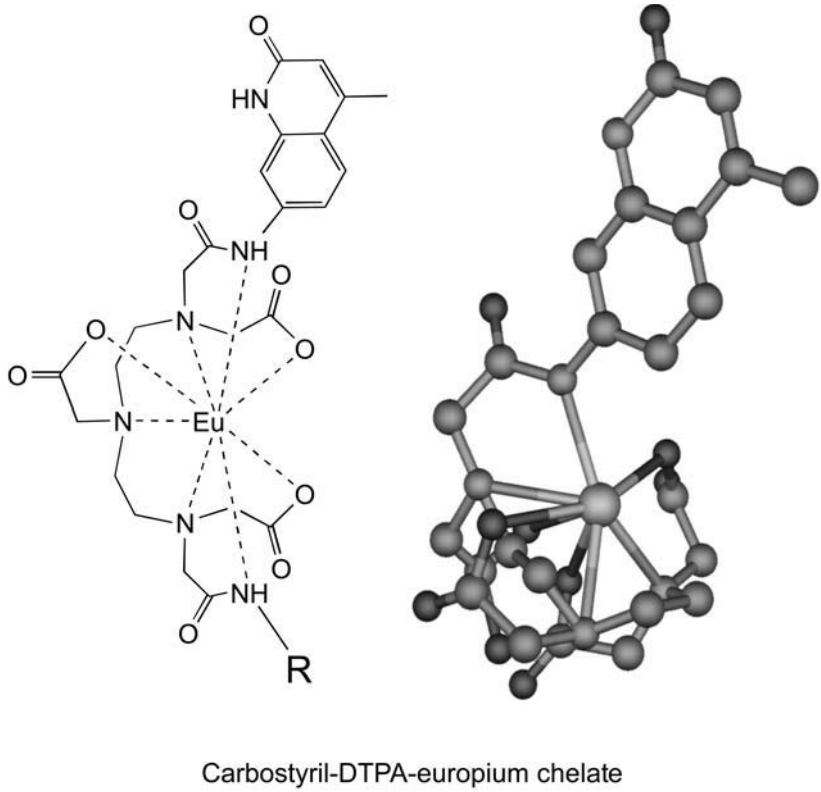


Figure 9.55 A carbostyril–DTPA europium chelator is a strong enhancer of lanthanide luminescence. The chemical structure and three-dimensional structure of the chelator are shown.

compounds via amide bond formation with the 7-amino group to a carboxylate (Figure 9.55). A carbostyril-DTPA derivative has intense luminescence for use in TR-FRET-based assays for drug discovery. The 7-aminoquinolinone structure is a common choice for designing fluorescent lanthanide chelates (Soini and Lövgren, 1987; Sammes and Yahioglu, 1996; Selvin, 2002, 2003). The synthesis of various carbostyril derivatives is described in Ge and Selvin (2004). The 7-aminoquinolinone group first is put onto DTPA by reaction with one of the chelator's anhydride rings. Linking the chelator to proteins or other molecules is done through the anhydride on the opposite end by forming an amide-linked spacer arm. In some cases, sulfonate groups or other hydrophilic components on the carbostyril structure can provide increased hydrophilicity for modifying biomolecules.

When used with europium or terbium ions, a carbostyril-based lanthanide chelate can be excited at 340 nm and provide sharp characteristic emission bands for transfer of energy to the appropriate acceptor fluor. Similar to the TMT chelator described previously, luminescence from terbium FRET signals well with Cy3 dyes and luminescence from europium can be used with APC or Cy5 dyes. Other fluorescent dyes that have similar excitation and emission ranges to these also can be used as acceptors in TR-FRET assays. For instance, terbium chelates can

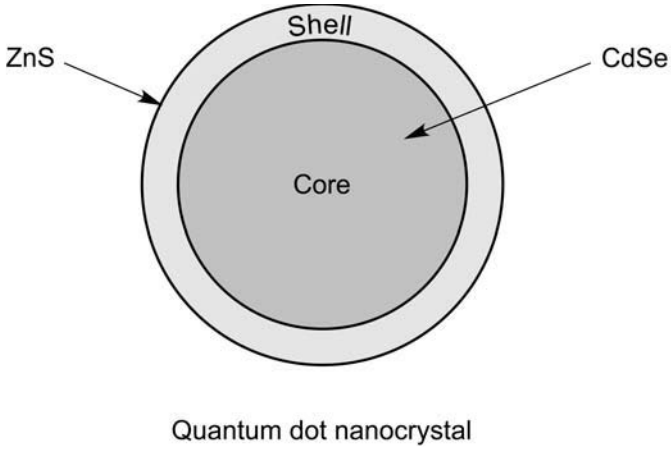


Figure 9.56 The structure of a typical QD nanocrystal includes a semiconductor alloy core surrounded by a shell consisting of a different alloy structure. Early QD compositions involved the use of CdSe core with a ZnS shell, but many different alloy compositions have been used and are possible.

be used with fluorescein and tetramethylrhodamine dyes, if the appropriate filters are used for measuring emission.

The potential for creating an expressed protein time-resolved luminescence system was realized in the development of a novel lanthanide-chelating complex derived from a metal binding peptide sequence. This sequence, called a lanthanide binding tag (LBT), can be used as a fusion tag in recombinant protein expression (Franz *et al.*, 2003; Nitz *et al.*, 2003; Wöhnert *et al.*, 2003; Lim and Franklin, 2004; Goda *et al.*, 2007). To create this luminescent tag, a peptide sequence of about 15–20 amino acids representing the basic structural domain of a calcium binding loop from calmodulin was mutated to coordinate tightly a terbium ion. Luminescence occurs through excitation in the UV of a nearby tryptophan residue with energy transfer to the chelated lanthanide. Recombinant mutagenesis of the original calcium binding domain resulted in optimizing the dissociation constant for terbium to within the nanomolar range, thus the metal ion is tightly associated with the peptide tag (Martin *et al.*, 2005). Recombinant proteins containing this small fusion tag can be detected using time-resolved fluorescence techniques. Such *in vivo* expression of a luminescent lanthanide tag can be used to study protein interactions using LRET or track individual proteins within cells (Sculimbrene and Imperiali, 2006).

10. Quantum Dot Nanocrystals

Properties of Quantum Dots

Quantum dots (QDs) are nanoparticles typically made of a semiconductor metal alloy arranged in a spherical crystalline core and capped with a shell consisting of a second metal alloy composition (Figure 9.56). The size of this raw core/shell construct is usually less than 10 nm in diameter or about the same sizes as many globular protein molecules. Upon exposure to light at the appropriate wavelength range, QDs are able to absorb a photon of energy, which results in

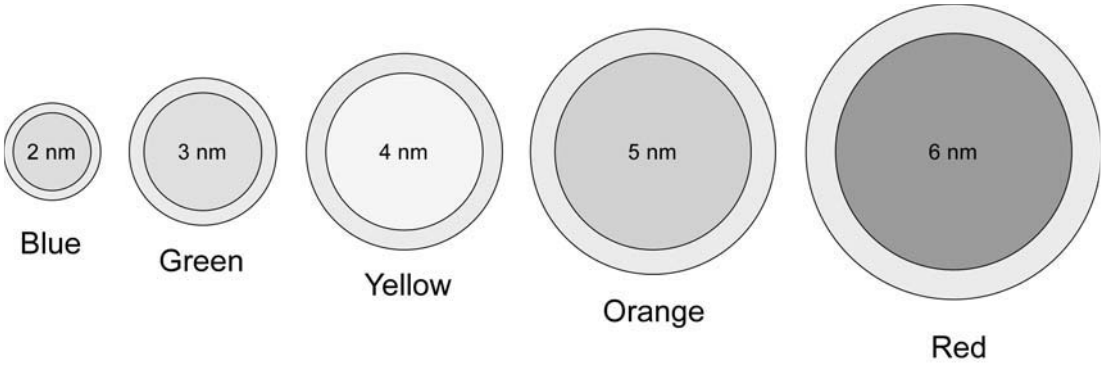


Figure 9.57 The size of a QD directly affects its emission wavelength. Careful control of nanocrystal diameter during the manufacturing process can result in discrete QD populations having emission properties ranging from the blue to the red region of the visible spectrum.

the excitation of an electron within the core. The excited electron is confined within the nanocrystal, because its core diameter is less than the exciton Bohr radius, which thus leads to quantum confinement. The shell structure aids in this confinement and prevents the electron from tunneling out of the core and escaping into the outer medium or undergoing non-radiative deactivation. The size and shape of a QD governs the discrete energy levels that the excited-state electron can attain within it, thus dots can be tuned to have desired electronic properties by careful adjustment of core diameter and composition. Upon return of the electron to its ground state, a radiative QD emits a photon of light, the wavelength of which is dependent on the alloy material type and its diameter (Alivisatos, 1996) (Figure 9.57).

As a result of their unique optical and electronic properties, particularly their ability to fluoresce at discrete wavelengths directly proportional to their sizes and material compositions, QDs have found use in many fields, including electronics, biology, medicine, and even cosmetics. The first attempts to modify their surface characteristics to make them water-soluble and biocompatible eventually led to their use as fluorescent labels for biomolecules in many applications (Rogach *et al.*, 1996; Bruchez *et al.*, 1998; Chan and Nie, 1998).

QDs have a number of advantages over other fluorescent molecules such as organic dyes, including: (1) resistance to photobleaching, which allows them to be imaged over long periods without loss of fluorescence; (2) narrow, nearly symmetrical emission peaks with no red-shift tail typical of organic fluors, thus creating a bright fluorescence signal at characteristic wavelengths; (3) a broad absorbance band, which increases almost exponentially toward shorter wavelengths with extremely high extinction coefficients (10^5–$10^7\,M^{-1}\,cm^{-1}$); (4) the ability to excite at a single wavelength an entire family of QDs having different emission characteristics, thus providing multiplexed assay capability; (5) the capacity to design QDs with emission characteristics ranging from the low visible wavelengths to well within the IR region; and (6) the potential for relatively high QY of fluorescence (0.65–0.95 for CdSe).

The emission properties of QDs can be adjusted based upon core diameter and nanoparticle composition. Nanoparticle diameters typically are carefully controlled during manufacture to be between 2 and 10 nm. In addition, the band gap energy or energy of fluorescence emission is inversely proportional to the diameter of the QD particle. Thus, the smaller the particle, the

more blue-shifted is its emission and the larger the QD, the more red-shifted is its emission bands. QDs also have an intrinsic color to their solutions that corresponds to the size of the particles and their fluorescence emission characteristics. However, to create a single particle population with a tight fluorescence emission pattern, the diameter of the particles must be controlled to well within a nanometer. The emission peak width is directly proportional to the size distribution of a particle population. This makes manufacturing reproducible QDs a constant challenge for most suppliers that rely on size to control fluorescence properties.

However, as opposed to the difficulty of tuning emission properties by particle diameter, QD alloy composition instead may be adjusted independent of size to control the wavelength of emission for a given particle population. In a QD having a concentration gradient composition, the concentration of an alloy of a first semiconductor gradually increases from the core to the surface of the particle, while the concentration of a second semiconductor gradually decreases from the core to the surface (Nie and Bailey, 2007). A third semiconductor type also may be added to fine-tune further the emission properties. By careful adjustment of these semiconductor concentration gradients, QD populations can be made having discrete emission properties without changing the particle size. Therefore, tuning QD spectral characteristics can be done using a single particle size and by making selective changes to the alloy composition. This avoids the difficulties in manufacturing particles of uniform size, because all particle populations can have the same size, but only vary in their relative semiconductor gradient concentrations to attain particles having discrete fluorescence character.

The material types making up the core of a QD also affect the range of emission wavelengths that can be attained. For common material types, the ranges of emission wavelengths that can be achieved by adjustment of particle diameter or composition are: CdSe = ~470–660 nm, CdTe = ~520–750 nm; InP = ~620–720 nm; PbS > 900 nm; and PbSe > 1,000 nm.

QDs have been made using a number of techniques. A common method to make bulk quantities of particles involves doing colloidal suspension synthesis in organic solvent with nucleation of semiconductor metals under high-temperature conditions (Murray *et al.*, 1993; Hines and Guyot-Sionnest, 1996; Dabbousi *et al.*, 1997). In one such process, a solvent such as octadecene is stirred at constant rate and heated to >300°C at which point solutions containing the semiconductor metals are injected. The metals at first decompose under high heat and then recombine to form alloys consisting of nanoparticle seeds, which grow to create the QDs. The reaction time determines the size of the nanoparticles and thus their spectral properties. Detergent molecules often are added to coat the resulting nanoparticles and prevent their aggregation during nucleation. Originally, the solvent and detergent molecule used for making QDs was TOPO (tri-*n*-octylphosphine oxide), which ends up coating the particles with the phosphine component interacting with the semiconductor surface and the alkyl chains pointing out into the organic solution (Figure 9.58). Other additives, such as stearic or oleic acid, function similarly. The raw particles thus prepared are hydrophobic and not dispersible in aqueous solution.

To use QDs in biological applications, the particles must be rendered biocompatible by coating with a hydrophilic layer that masks the surface, thus preventing aggregation and nonspecific binding. This is not a trivial problem, as the successful commercialization of QDs for biomolecule labeling took at least 5 years from the time the first two papers appeared in Science describing water-soluble particles for bioconjugation (Bruchez *et al.*, 1998; Chan and Nie, 1998). The fact is, these early particles were not very soluble in aqueous environments and tended to clump together or bind nonspecifically with biomolecules.

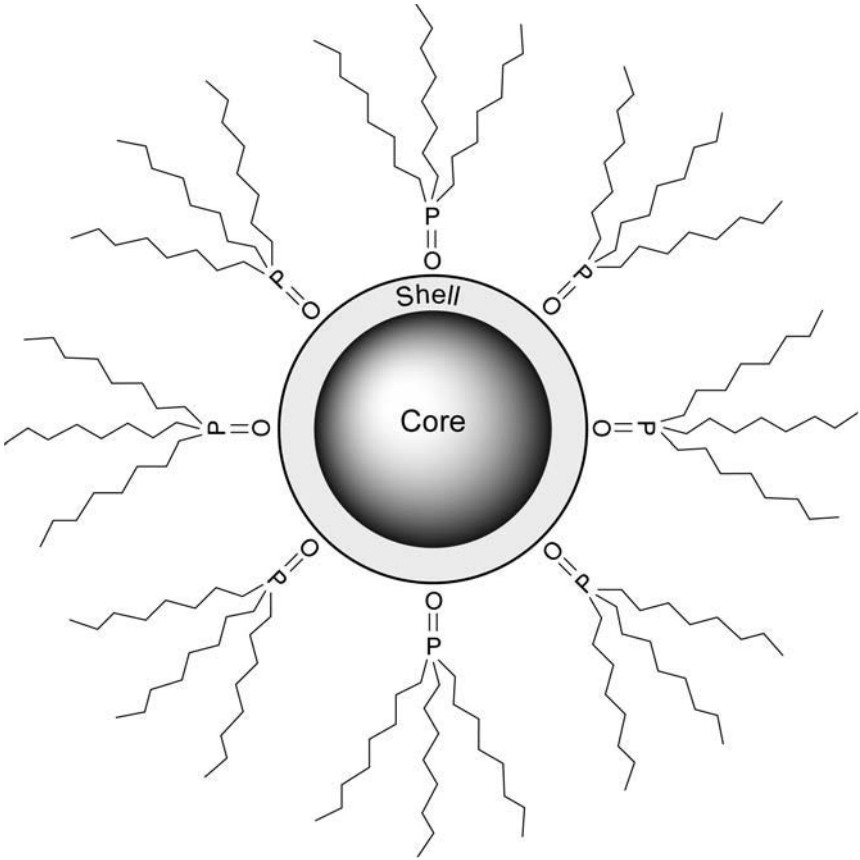


Figure 9.58 QDs made using the TOPO process typically have a layer of these molecules associated with their outer surface. The TOPO groups must be displaced and replaced by water-soluble groups to provide biocompatibility for bioconjugation purposes.

The initial modifications done to covalently link molecules to QDs need to displace the TOPO or detergent coating on the raw nanocrystal surface with a new organic derivative imparting water solubility. The first attempts at making biocompatible QDs all involved the use of simple monothioacids, such as thioacetic acid, which can link to the shell through thiol dative bonding and provide a terminal carboxylate for further conjugation (Figure 9.59). However, monothiol linkers easily can oxidize back off QDs and leave behind surface gaps, which become hydrophobic sites for particle clumping and nonspecific binding to biomolecules. A better approach is to use a dithiol compound, which forms two dative bonds per linker on the QD surface. This makes the linkage to the QD resistant to oxidation and prevents nonspecific surface gaps from forming. One such dithiol compound that is particularly useful is dihydrolipoic acid (DHLA), which contains a carboxylate group on the other end. QDs modified with DHLA subsequently can be modified with polyethylene glycol (PEG) groups to provide increased hydrophilicity of the surface or directly linked to proteins via electrostatic

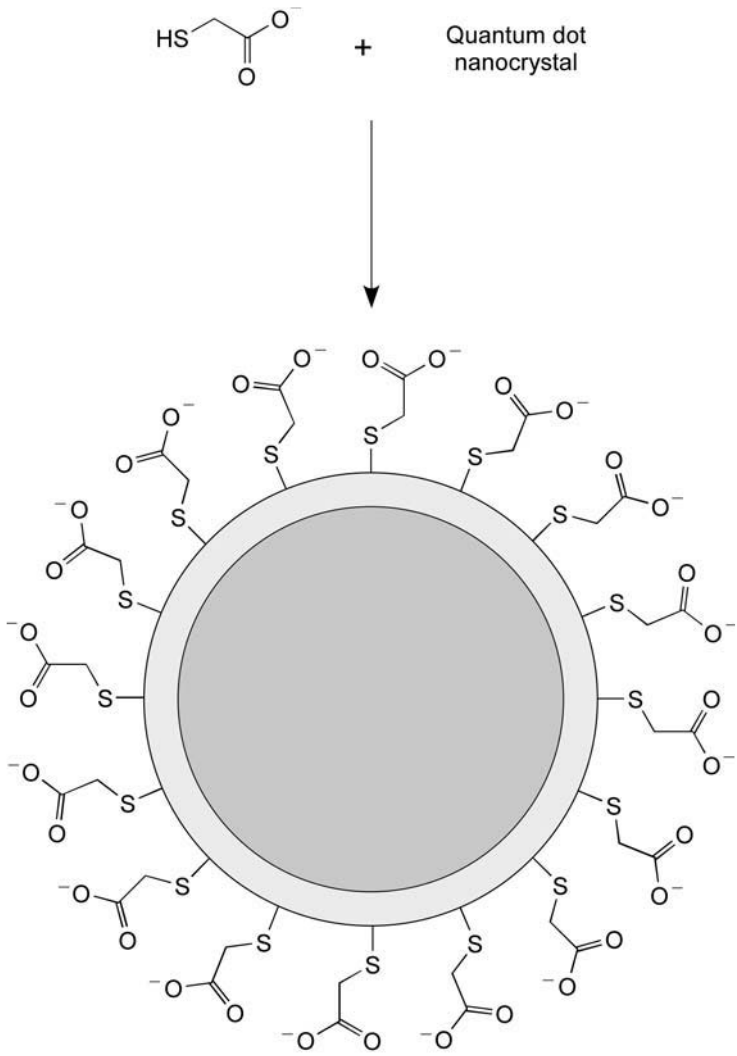


Figure 9.59 One of the first methods of preparing water-soluble QDs was to use thioacetic acid modification of the nanocrystal surface. This resulted in a negative charge on the surface of each dot that provides like charge repulsion of particles suspended in aqueous solution. The carboxylate group also could be used for conjugation with amine-containing molecules.

interactions or through an EDC-mediated reaction (Mattoussi *et al.*, 2000; Uyeda *et al.*, 2003; Medintz *et al.*, 2004; Anikeeva *et al.*, 2006; Clapp *et al.*, 2006).

The negative charge character of DHLA-modified QDs has been used to link noncovalently positively charged proteins, such as avidin (Goldman *et al.*, 2002a) or a recombinant protein containing a positively charged fusion peptide. In this regard, the highly positive leucine zipper

peptide has been used as a fusion tag (Goldman *et al.*, 2002b) as well as a penta-histidine peptide tag (Medintz *et al.*, 2003). Combinations of positively charged fusion proteins and avidin also have been used to control the resultant biotin binding density on a DHLA-QD surface for use in live cell imaging (Jaiswal *et al.*, 2004).

One major advantage of DHLA modification is that the diameter of the QD remains as small as possible, while still creating a water-soluble particle. QDs of <10 nm diameter have been created using this process and were successfully used to image intracellular proteins (Jaiswal *et al.*, 2003).

Another type of simple surface modification involves the noncovalent coating of the QDs with detergents or lipids. The hydrophobic tails of these molecules bind to the QD particle, while the hydrophilic portions interact with the aqueous phase and render the dots dispersible. Still other surface modification schemes use polymeric coatings containing multiple binding points to the QD, thus eliminating the possibility for leaching. Coatings containing PEG spacers also can be used to create a highly hydrophilic layer on top of the semiconductor surface. All of these modification strategies provide QDs that are water-soluble (or dispersible and stable in suspension) and that contain functional groups for covalent attachment of proteins or other affinity molecules.

Masking the surface of semiconductor QD particles also can be done by adding another inorganic layer to the outer shell alloy structure. This layer can take the form of a silica coating formed by the reaction of a silane derivative with the shell (Darbandi and Nann, 2005). For instance, a pure silica surface can be created by controlled polymerization of the raw nanocrystals with tetraethyl orthosilicate (TEOS), which forms a siliceous sphere with silanol groups on the outer surface. Another organosilane derivative that is appropriate for use with metallic particles is mercaptopropyl-tris-hydroxy-silane. The thiol groups on the silane compounds datively bind to the surface while the hydroxy-silane groups polymerize to form a new silica coating. The resultant silanol-containing surface then can be functionalized using other organosilane compounds containing functional groups or reactive groups for further conjugation with biomolecules (see Chapter 13 for organosilane reagents and protocols). The only disadvantage of this approach is the increasingly greater particle diameter that results from building successive layers on the initial QD core, which may inhibit their use for probing within cells or tissues (Figure 9.60).

Water-soluble QDs now are available from a number of manufacturers (Invitrogen, Evident Technologies, and Crystalplex). Each supplier uses their own proprietary methods of surface pacification to create biocompatible particles. Even coated QD clusters are available that contain hundreds of particles bound together in a polymer matrix (Crystalplex). These form intensely bright labels for biomolecules, because the nanocrystals do not quench when clustered together at high density.

Most QD surfaces for biological applications contain negatively charged carboxylates for conjugation with amine-containing molecules via a carbodiimide reaction with EDC and (sulfo)NHS (Chapter 3, Section 1). The negative charges on the QD surface prevent particle aggregation through like charge repulsion. An alternative method of creating water dispersible dots is to form a hydrophilic coating that carries along with it a layer of hydration consisting of hydrogen-bonded water molecules. This often is done using hydroxylic polymers or PEG modifications. This too prevents aggregation due to the high energy needed to remove the bound water layer.

QDs have been used successfully in many biological applications, which exploit their best properties of brightness, photostability, and multiplex capability. There are many publications

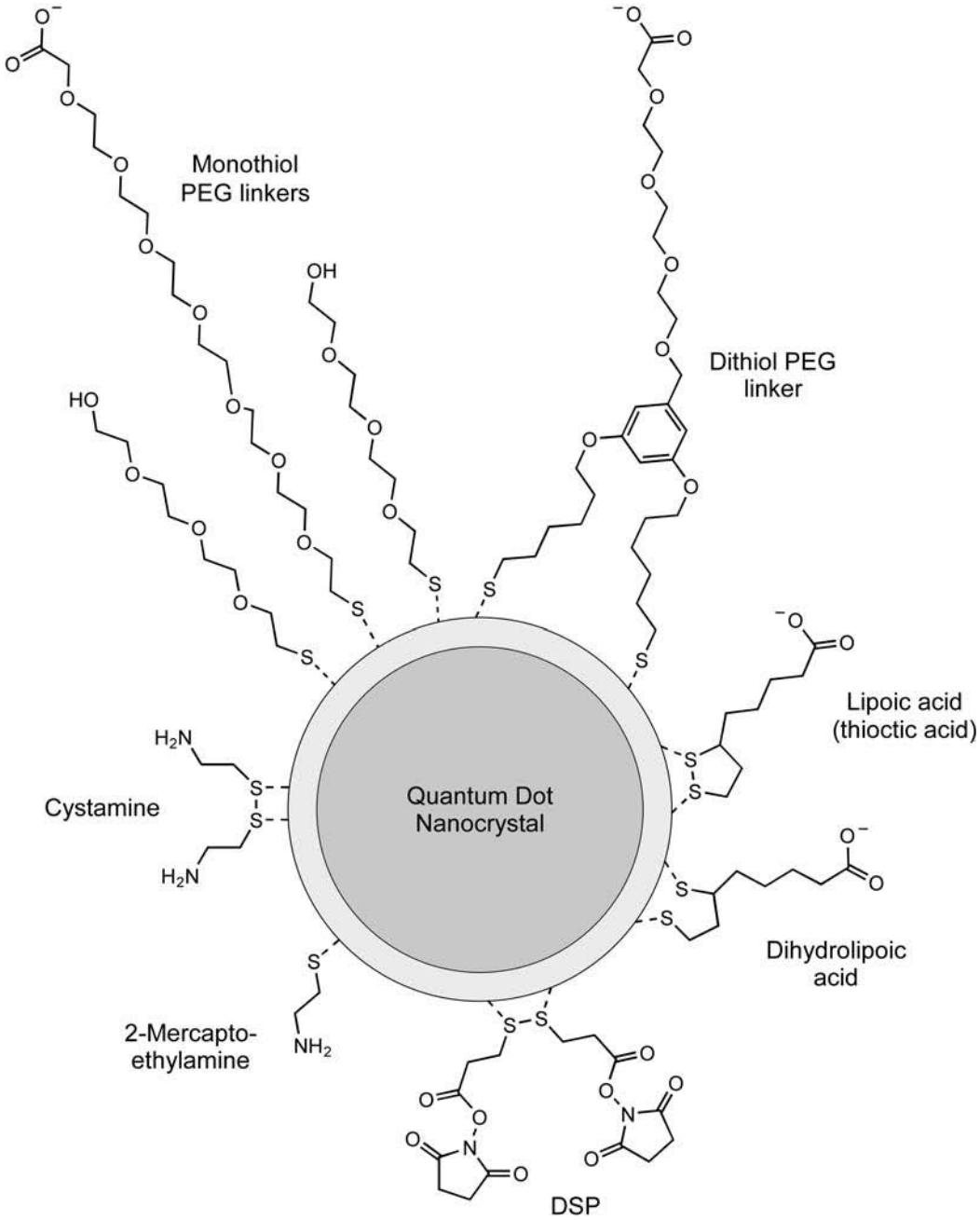


Figure 9.60 Many different thiol-containing linkers can be used to prepare water-soluble QDs. The monothiol compounds suffer from the deficiency of being easily oxidized or displaced off the surface, thus creating holes for potential nonspecific binding. The dithiol linkers are superior in this regard, as they form highly stable dative bonds with the semiconductor metal surface that do not get displaced. The PEG-based linkers are especially effective at creating a biocompatible surface for conjugation with biomolecules.

that use QDs for in cell or whole organism-based imaging, including tracking of targets within cells (Dahan *et al.*, 2003), gene localization within chromosomes (Xiao and Barker, 2004), embryo developmental monitoring (Dubertret *et al.*, 2002), tumor imaging *in vivo* (Gao *et al.*, 2004), and multiplexed imaging and assays (Medintz *et al.*, 2003; Wu *et al.*, 2003), including FRET signaling (Han *et al.*, 2001). For a review on the use of QDs for cancer imaging and treatment, see Vashist *et al.* (2006).

However, QD labels still are not without potential problems. Although the raw dots typically are less than 10 nm in diameter, the addition of thick surface layers for biocompatibility and conjugation can increase the hydrodynamic radius considerably. Most particles with polymer coatings are in the 20–50 nm range in diameter, which often limits their use for cell-based detection due to their inability to easily penetrate cells and diffuse freely to intracellular targets. Most cell imaging applications with this type of QD involve cell-surface staining or transport within cells by endocytosis, which limits particle access to other areas within the cell. In addition, nonspecific binding still plagues some QD probes when used with complex biological samples.

Another potential deficiency of QDs is the toxic nature of their metallic composition. Most particles contain at least one known toxic metal (e.g., cadmium) or contain alloys with unknown toxilogical properties. Cadmium-based QDs exposed to UV light for long periods release cadmium ions, which are highly toxic to cells (Derfus *et al.*, 2004). The initial proposal that QDs would be ideal as fluorescent probes for *in vivo* diagnostic imaging may not be fully realized due to the potential for heavy metal toxicity in humans. Even the use of QDs for *in vitro* research purposes must be done with care, as the solutions should be regarded as hazardous waste and disposed of according to standards for handling heavy metal contaminated solutions.

Another potential difficulty with using QDs relates to their special spectral characteristics. Excitation of QDs optimally occurs in the low region of the spectrum, typically below 400 nm, while emission usually is measured in regions that can be hundreds of nanometers away from the excitation wavelength. Unfortunately, many instruments for imaging or fluorimetry still don't contain lasers and filter sets that exactly match QD excitation and emission patterns. Most instruments in use today initially were designed for organic fluors with matched filter sets for such common dye derivatives as fluorescein, rhodamine, and the cyanine dyes. Using QDs with these instruments may mean exciting at a non-optimal, higher wavelength than is recommended to obtain full brightness.

However, the most important issue with QD fluorescence is their tendency to blink or to be completely dark and not fluoresce at all. Individual QDs can undergo an on/off cycle that results in a dark period of no light emission after a photon has been emitted (Nirmal *et al.*, 1996; Efros and Rosen, 1997). This blinking can be observed on the order of milliseconds and can be problematic if imaging at the single dot resolution or if using QDs for flow cytometry purposes is important. When imaging a larger population of QDs, the problem of blinking will be overcome by the fact that at any given moment many of the particles will be emitting light and thus contributing to the overall signal. Blinking will lower the apparent QY for the combined QD population, but it won't eliminate signal entirely. In addition, certain solution additives used at relatively high concentration (i.e., 100 mM DTT or 2-mercaptoethanol) may serve to limit the blinking phenomena (Hohng and Ha, 2004).

A more severe issue with QDs, however, is the problem of dark or nonradiant dots in aqueous solution. Yao *et al.* (2005) documented that a significant fraction of commercially available

QD particles in a population can be entirely dark. The nonradiant properties probably are due to defects in their nanocrystalline structure that occurred during manufacture. The percentage of dark dots varies for each sample, but they can represent 44–47 percent of all dots in a population. Thus, the use of QD conjugates for biomolecule imaging may mean that nearly half of all particles in an assay do not contribute to the resultant fluorescence signal.

Conjugation to QDs

Many antibodies, proteins, and other targeting or affinity ligands have been conjugated to QDs for biological applications. Antibodies to tumor markers have been used to image cancer cells *in vivo* (Tada *et al.*, 2007), fluoroimmunoassays have been developed using antibody-conjugated QDs (Goldman *et al.*, 2005), peptide–QD conjugates have been made to target proteins *in vivo* (Ness *et al.*, 2003), antibody–QD conjugates containing tumor toxic agents have been designed to image and kill tumor cells (Gao *et al.*, 2004), and sugar–QD conjugates have been made to detect carbohydrate binding proteins (Babu *et al.*, 2007).

The conjugation of proteins and other molecules to QDs involves standard coupling reactions with the added caveat related to the potential difficulties of working with particles. Many of the coupling strategies described in Chapter 14 for dealing with nanoparticles and microparticles are valid for use with QDs, but it is best when using commercially available particles to pay close attention to the manufacturer's suggested protocols.

When designing a protein–QD conjugate, it is also important to consider the optimal number of proteins to be coupled per particle. In some applications, a low ratio of protein-to-QD may result in the highest signal for fluorescence detection. This often is the case for antibody–QD conjugates where two to three antibodies coupled per particle are sufficient to target antigens. However, other applications may require a high density of protein on the QD surface. For instance, as a result of investigations into the interaction of CD8 with HLA A2 complexes, it was found to be important to create multiple contacts between the HLA molecules and the CD8 molecules on cell-surface membranes. Therefore, up to 12 HLA A2 complexes per QD were coupled (the maximal possible to fit on the particle surface) to assure the greatest interaction potential with cells (Anikeeva *et al.*, 2006). Additionally, when preparing (strept)avidin–QD conjugates, it may be desirable to maximize the biotin binding ability of the complex to interact with as many biotinylated molecules as possible. Commercial streptavidin–QD conjugates typically have at least 5–10 proteins coupled per particle. Since each QD conjugate will have its own unique use, the conjugation process should be optimized to perform best in its intended application.

As is the case with most nanoparticles, buffer and salt compatibility should be taken into account when working with QDs. Small charged particles maintain colloidal stability by like charge repulsion, which prevents aggregation by van der Waals or hydrophobic attraction. Any salt or buffer constituents that neutralize or eliminate the surface charge on the QDs will cause clumping or precipitation. Some buffer additives also will cause loss of fluorescence intensity and should be avoided in concentrations above a certain level. Consult the supplier's guidelines for buffer suggestions to maintain particle stability.

The following methods are based on those cited in the literature or in company instruction manuals for coupling molecules to fluorescent nanoparticles. The coupling of unique proteins or other molecules to QD surfaces may need further optimization of reactant ratios as well as time and temperature to obtain the best conjugates.

Conjugation of Proteins to QDs Using EDC

Carbodiimide coupling to carboxylate-containing QDs usually involves the use of EDC in a single-step or two-step process to form an amide bond. If a one-step reaction is done, the QD is activated with EDC in the presence of an amine-containing molecule, such as a protein. Many protocols use this method, but it can result in protein polymerization in addition to coupling, because proteins contain both carboxylates and amines. A two-step protocol results in better control of the reaction (Figure 9.61). In the first step, EDC is used in the presence of sulfo-NHS to activate the carboxylates on the particles to intermediate sulfo-NHS esters. After a quick separation step to remove excess reactants, the activated QDs are added to the protein solution to be coupled. This then results in amide bond formation without polymerization of the protein in solution. See Chapter 3, Section 1 and Chapter 14, Section 1 for additional information on this process.

The following protocol describes the coupling of amine-containing proteins to carboxylated QDs using a single-step EDC reaction, as recommended by several manufacturers.

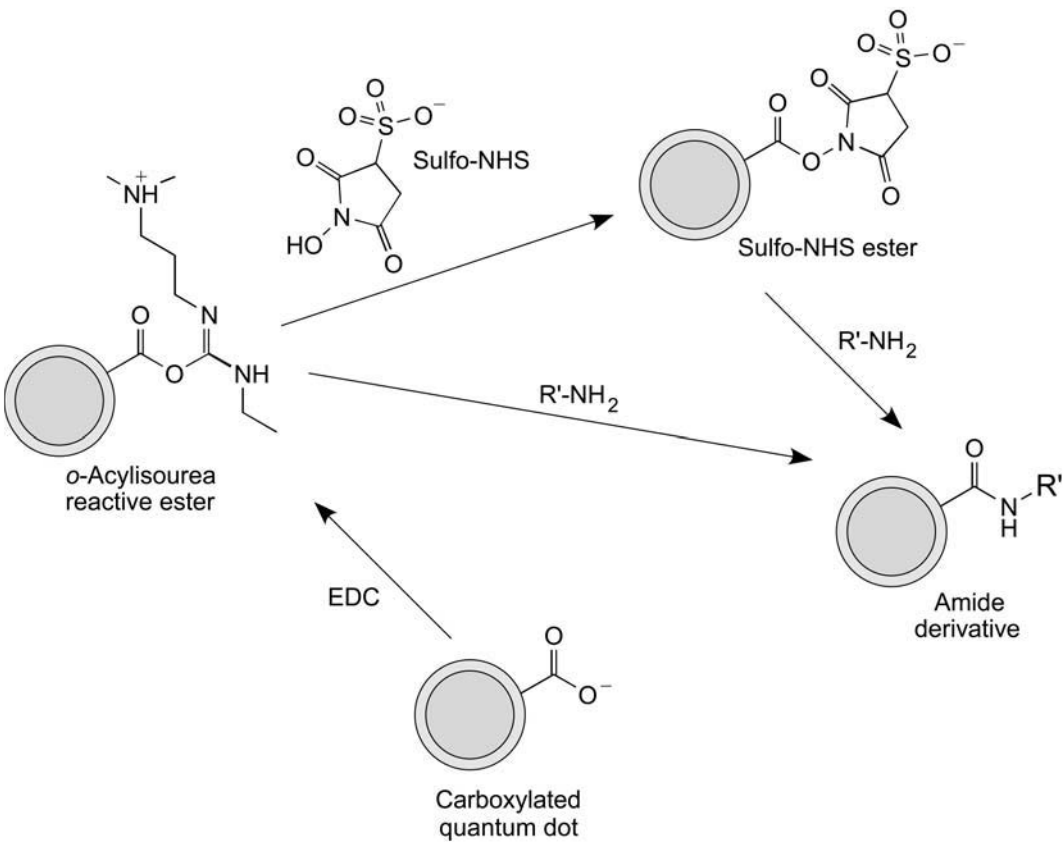


Figure 9.61 QDs containing carboxylate groups can be coupled to amine-containing proteins or other molecules using the EDC/sulfo-NHS reaction to form amide bond linkages. The intermediate sulfo-NHS ester is negatively charged and will help maintain particle stability due to like charge repulsion between particles.

Protocol

1. Prepare a carboxylated QD solution in 10 mM sodium borate, pH 7.4 (reaction buffer), at a concentration of 1 μM. The supplier of QDs usually will provide the reagent concentration as a molar quantity, which treats each particle as though it was a single molecule. A typical QD solution as obtained from a manufacturer may be about 8 μM starting concentration.
2. Dissolve the protein to be conjugated to the QD in reaction buffer at a concentration of 1–10 mg/ml.
3. Add a quantity of the protein solution to the QD solution with mixing to obtain the desired molar excess of protein over the concentration of nanoparticles. Using a 1- to 20-fold molar excess typically works well, but optimization should be done to determine the best ratio for a particular application.
4. Prepare a solution of EDC in water at a concentration of 10 mg/ml. Immediately add 57 μl of this solution to the protein/QD solution. Mix well.
5. React for 2 hours at room temperature with gentle mixing.
6. Filter the solution through a 0.2 μm filter (low protein binding type) to remove any precipitated protein or particles.
7. Separate excess protein from the protein–QD conjugate by use of ultrafiltration spin columns using an exclusion limit appropriate for allowing passage of the unconjugated protein, but retention of the protein–QD conjugate. For proteins of molecular weight below 100 kD, a membrane of this cutoff will work well.

QD nanoparticles containing carboxylate groups also may be reacted in a two-step EDC/sulfo-NHS reaction to couple proteins and other molecules containing both amines and carboxylates. This type of reaction is designed to remove excess EDC activating agent before addition of protein, so protein polymerization cannot occur.

Protocol

1. Prepare a QD solution in 25 mM PIPES buffer, pH 7.0 (reaction buffer), at a concentration of 100 μg/ml.
2. Prepare a protein solution in reaction buffer at a concentration of 1 mg/ml.
3. Prepare a solution of 20 mM EDC, 50 mM sulfo-NHS in water immediately before use.
4. To each ml of QD solution, add 50 μl of the EDC/sulfo-NHS stock solution. Maintain the pH at 7.0 by the addition of base, if necessary. Small volume reactions may be controlled using a pH stat.
5. React for 10 minutes at room temperature with gentle mixing.
6. Add 1.4 μl of 2-mercaptoethanol to each ml of the reaction mixture to quench excess EDC. Sonicate the QD solution several times to maintain particle dispersion.
7. Add 5 μl of the protein solution to each 100 μg quantity of activated QDs.
8. React for 60 minutes at room temperature with mixing.
9. Remove excess reactants and block remaining sulfo-NHS ester sites by dialysis against 50 mM Tris, pH 7.4. Use a membrane cutoff appropriate to allow passage of the protein being coupled, but retention of the protein–QD conjugate.

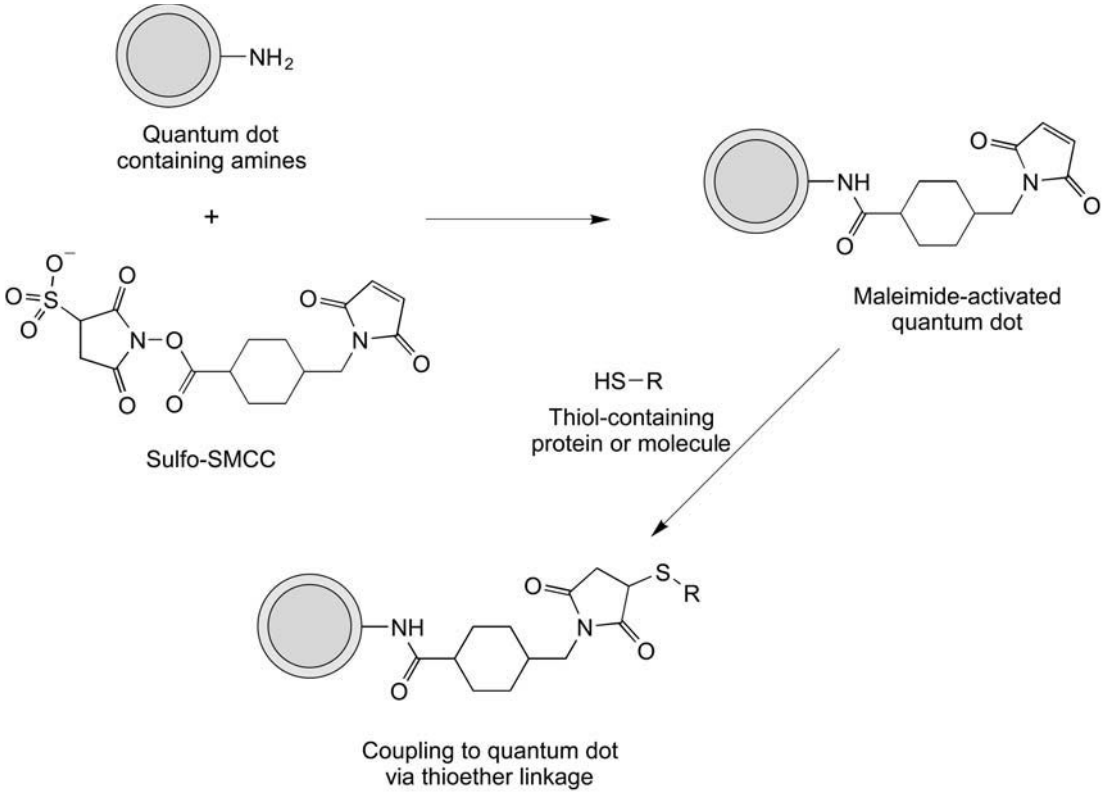


Figure 9.62 Sulfo-SMCC can be used to conjugate amine-containing QDs with thiol-containing proteins or other molecules using a two-step coupling procedure.

Conjugation to QDs Using Sulfo-SMCC

Sulfo-SMCC is a heterobifunctional crosslinking agent containing an amine-reactive NHS ester on one end and a thiol-reactive maleimide group on the other end (Chapter 5, Section 1.3). Amine-containing QDs may be activated with sulfo-SMCC to contain sulfhydryl-reactive maleimides for conjugation with thiol-containing proteins or other molecules (Figure 9.62). The following protocol illustrates this process.

Protocol

1. Prepare a 200 µl solution of amine-containing QDs at a concentration of 2.5 µM in 50 mM sodium phosphate, pH 7.4 (reaction buffer). This represents 0.5 nmole of QD in 200 µl buffer.
2. Add 1 mg of sulfo-SMCC to the QD solution with mixing to dissolve the crosslinker.
3. React for 60 minutes at room temperature with mixing.
4. Remove excess crosslinker and reaction by-products by gel filtration on a desalting resin. The QD fraction is identified by its characteristic fluorescence. This operation should be

done quickly to limit the degree of maleimide hydrolysis. The resulting particles contain reactive maleimide groups for coupling to a thiol-containing protein or other molecule.

5. Prepare a protein or antibody containing an available thiol group in reaction buffer at a concentration of 1–10 mg/ml. Add a quantity of this protein solution to the purified nanoparticle suspension to attain the desired molar excess of protein over the concentration of activated QDs. Typically, a 1- to 20-fold molar excess of protein over the QD concentration works well. The optimal amount of protein to be added should be determined experimentally by considering the best performance of the fluorescent conjugate in its intended application. Tada *et al.* (2007) created a monoclonal anti-HER2 antibody–QD conjugate at a level of three antibodies per nanoparticle to target tumors in mice. Creating thiol groups on proteins or peptides may be done from disulfides by reduction. Alternatively, a thiolation reagent may be used to add thiols to the protein surface for coupling (see the protocols in Chapter 1, Section 4.1).
6. React with mixing for 2 hours at room temperature. At the completion of the reaction, cysteine may be added at 50 mM to block excess maleimide-reactive sites.
7. Purify the QD conjugate using gel filtration or ultrafiltration using a micro-spin device. The use of a molecular weight cutoff for gel filtration that will accommodate both the conjugate and the not-coupled protein is appropriate (i.e., Superdex-200 resin). For ultrafiltration, use a membrane cutoff that will retain the conjugate, but permit the not-coupled protein to pass through.

10

Bifunctional Chelating Agents and Radioimmunoconjugates

Monoclonal antibodies provide extremely high antigen specificity that can be useful as cancer targeting and therapeutic reagents *in vivo* (Waldmann, 1991). Radiolabeled monoclonals are currently undergoing developmental clinical trials for their use in the diagnosis or treatment of cancer. The antigen-binding specificity of the radioimmunoconjugate provides the targeting capability to localize in tumor sites, while the associated radiolabel provides cytotoxic properties or detectability for imaging applications (Schlom, 1986).

Iodine-131 was among the first radioactive isotopes used for radioimmunoconjugate preparation (Order, 1982; Regoeczi, 1984). Since the earliest studies on the efficacy of radiotherapy, additional isotopes have been employed, such as iodine-125, bismuth-212, yttrium-90, yttrium-88, technetium-99 m, copper-67, rhenium-188, rhenium-186, galium-66, galium-67, indium-111, indium-114 m, indium-115, and boron-10.

There are several methods commonly used to label monoclonal antibodies with radionuclides. In a direct labeling process, a radioactive atom is attached to functional groups on the antibody without the use of an intervening chemical spacer. For instance, radioiodination can be done through modification of tyrosine side chains using established techniques and reagents (Chapter 12). Another direct method uses indigenous sulfhydryl groups or those formed through disulfide reduction to couple covalently certain metal nuclides (Holmberg and Meurling, 1993; Ranadive *et al*., 1993). Thiolation reagents also can be used in this regard to create the requisite —SH groups by modification of other protein functional sites (Joiris *et al*., 1991) (Chapter 1, Section 4.1).

Indirect methods of protein labeling with radiolabels utilize organic compounds able to chelate metal ions in a coordination complex. Bifunctional chelating agents (BCAs), as they are called, contain a reactive group for coupling to proteins or other molecules and a strong metal-chelating group for complexing certain radioactive metals. Their extensive use with monoclonal antibodies that are able to target specific cellular antigens has resulted in important radiopharmaceutical applications for the diagnosis and treatment of cancer (Wessels and Rogus, 1984; Meares, 1986; Otsuka and Welch, 1987; Hnatowich, 1990; Liu and Wu, 1991; Subramanian and Meares, 1991). The BCAs may be loaded with the radioactive metal before or after their conjugation with a monoclonal antibody (Frytak *et al*., 1993). If they are loaded with radionuclides prior to modifying the antibody, the BCA–metal pair is called a preformed complex (Kasina *et al*., 1991).

The following sections describe the major methods and BCAs used to create radioimmunoconjugates.

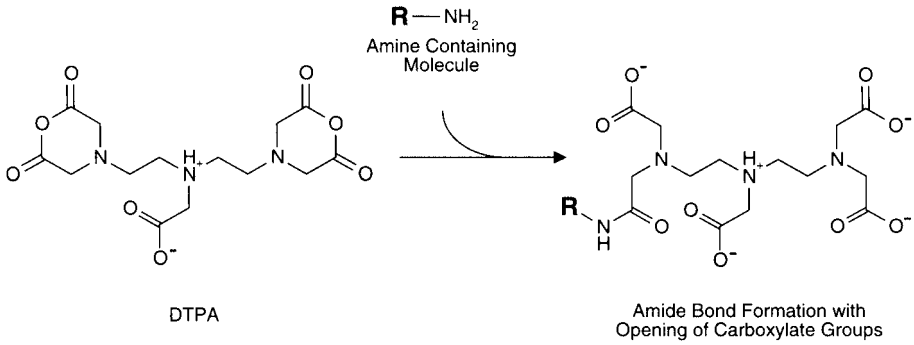
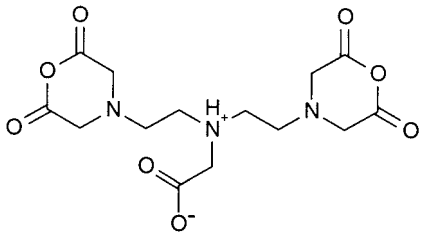


Figure 10.1 DTPA reacts with amine-containing molecules via ring opening of its anhydride groups to create amide bond linkages. The potential also exists for both anhydride groups to react and cause crosslinking of modified molecules, which is undesirable.

1. DTPA

DTPA is diethylenetriaminepentaacetic anhydride, a BCA containing two amine-reactive anhydride groups. The compound reacts with N-terminal and ε-amine groups of proteins to form amide linkages. The anhydride rings open to create multivalent, metal-chelating arms able to bind tightly metals in a coordination complex (Figure 10.1) (Hnatowich *et al.*, 1982). Metal-chelate bonds are created through dative interactions with the unshared pair of electrons on each oxygen and nitrogen atom on DTPA, thus creating the potential for eight coordination sites with metal ions (from 3 nitrogens and 5 oxygens).

Optimal reaction conditions for antibody modification with DTPA are neutral to slightly alkaline pH environments containing no extraneous amines. A pH of 7–8 may be used with buffering provided by phosphate or bicarbonate buffers at 0.1 M. Since two anhydride groups are present on each DTPA molecule there is potential for creating crosslinks between two amine-containing molecules. Conjugation of antibody through DTPA crosslinks may be a major reason some immunoglobulins lose antigen-binding activity after modification (Lanteigne and Hnatowich, 1984). Optimization of the amount of protein present and the quantity of DTPA added to the reaction may have to be done to avoid this type of crosslinking and polymerization.



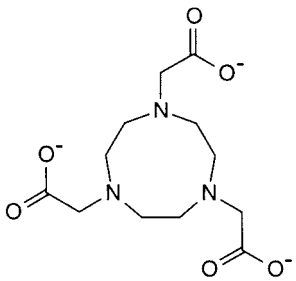
DTPA
Diethylenetriamine-pentaacetic anhydride
MW 357. 33

DTPA also can be used to modify amine-containing polymers, such as poly-L-lysine, to create a chelating polymer possessing multiple metal-binding sites (Trubetskoy *et al.*, 1993). Subsequent polymer modification of antibodies provides much higher radioactivity per molecule than if DTPA is directly coupled to the protein. Such chelating polymers can introduce into proteins as much as 100 DTPA residues able to complex radiolabels per each 55,000 Dalton poly-L-lysine chain attached (Torchilin *et al.*, 1993). Directed coupling to the antibody through only the N-terminal of the poly-L-lysine chain limits the modification to a single point along the polymer, thus avoiding crosslinking or multi-site attachment that can affect antibody activity (Slinkin *et al.*, 1991). This approach can dramatically increase the radioactivity level at tumor sites, thus increasing cytotoxicity or enhancing imaging capability.

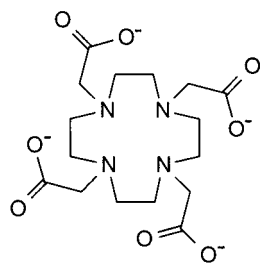
While DTPA has been used extensively as a BCA to prepare radiopharmaceutical reagents, newer metal chelators such as those discussed below may show greater promise for *in vivo* applications.

2. DOTA, NOTA, and TETA

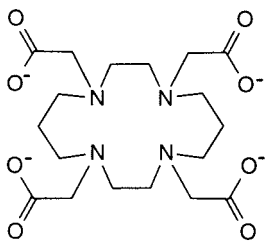
DOTA is 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid, a chelating ring structure containing 4 acetic acid carboxylate groups off the 4 nitrogens of its 12-atom cyclic structure. C- or N-functionalized derivatives of this basic structure produce a BCA capable of modifying proteins and binding radioactive metal ions in strong coordination complexes of up



NOTA
1,4,7-Triazacyclononane-
N, N', N''-triacetic acid



DOTA
1,4,7,10-Tetraazacyclodo-
decane-N, N', N'', N'''-tetraacetic acid



TETA
1,4,8,11-Tetraazacyclotetra-
decane-N, N', N'', N'''-tetraacetic acid

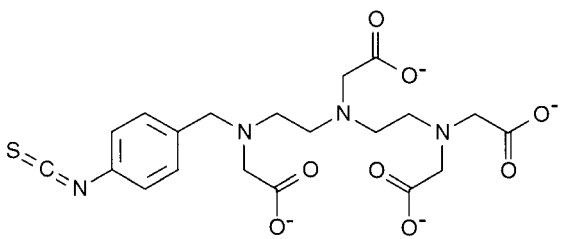
to eight dative bonds (Cox *et al*., 1990; Renn and Meares, 1992). Perhaps the simplest method of DOTA functionalization is through modification of one of its carboxylates to contain a short spacer terminating in a reactive group capable of being coupled to proteins (Li and Meares, 1993). However, modification of carbons on its cyclic backbone also is possible (Brechbiel *et al*., 1993). Complexes of metal ions with DOTA have been studied in detail (Sherry *et al*., 1989; Aime *et al*., 1992).

A similar BCA to DOTA is NOTA, which is 1,4,7-triazacyclononane-*N*,*N*′,*N*″-triacetic acid. NOTA contains a smaller ring structure in comparison to DOTA and only has 3 chelating carboxylate groups and 3 nitrogens, creating a maximal coordination potential for six dative bonds with metal ions. Synthesis of functional derivatives can be done through C- or N-modifications, creating reactive groups able to couple to proteins and other molecules (Cox *et al*., 1990). Cox *et al*. (1990) have prepared an (S)-lysine derivative of a benzamide-protected, C-substituted NOTA. Since coupling antibodies with NOTA through an amide linkage leaves only 2 free carboxylic acids, potentially making the reagent pentadentate for chelating purposes, metal complexes formed with this reagent may have lower stability than those containing greater numbers of chelating groups.

A third compound in the same category as DOTA and NOTA is TETA, which is 1,4,8,11-tetraazacyclotetradecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid. TETA contains a larger ring structure than the other two BCAs and has four chelating carboxylate groups and 4 nitrogens. Similar to the other two chelators, C- and N-functionalized derivatives can be prepared with TETA (Brechbiel *et al*., 1993). A *p*-bromoacetamidobenzyl-TETA derivative could be used to label antibodies through sulfhydryl groups and securely bind radioactive copper for probing biological systems *in vivo* (Moi *et al*., 1985).

3. DTTA

DTTA is *N*-(*p*-isothiocyanatobenzyl)-diethylenetriamine-*N*,*N*′,*N*″,*N*‴-tetraacetic acid. This BCA contains four carboxylate groups and 3 nitrogens that can hold metals tightly in a coordination complex of seven dative bonds. The compound is especially good at chelating lanthanide-series elements, such as europium, samarium, terbium, and dysprosium. Unlike the previous BCAs which are used to prepare radiopharmaceutical reagents, this one is used primarily for complexing metals to form fluorescent probes for time-resolved fluoroimmunoassays



DTTA
N^1-(p-isothiocyanatobenzyl)-diethylene-triamine-N^1, N^2, N^3, N^3-tetraacetic acid

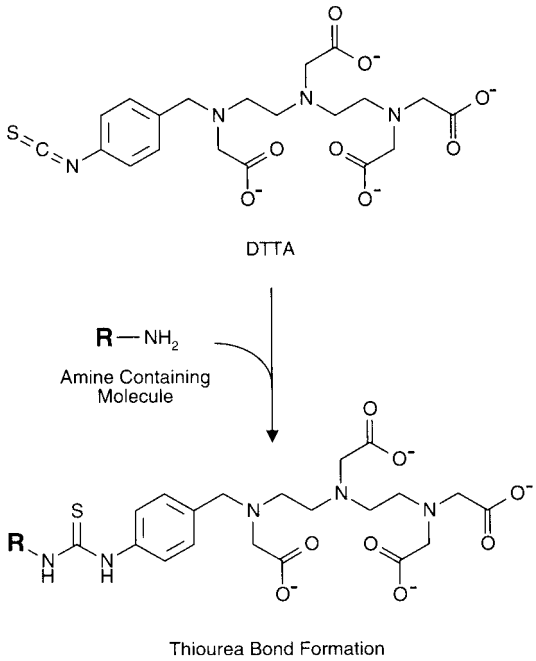


Figure 10.2 The isothiocyanate group of DTTA can react with amine-containing molecules to form isothiourea bonds.

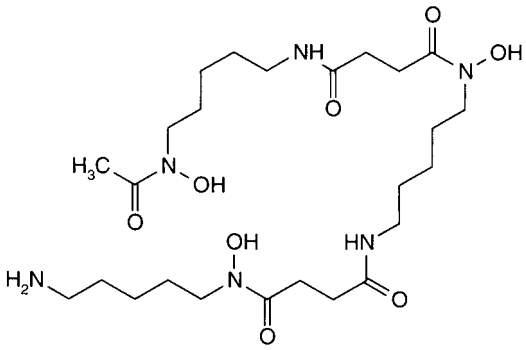
(Hemmila, 1988) (see Chapter 9, Section 9). The most commonly used lanthanides for this purpose are europium (Eu^{3+}), terbium (Tb^{3+}), and samarium (Sm^{3+}). Proteins modified with DTTA and complexed with lanthanide metal ions form the basis for unique fluorescent probes possessing long lived signals upon excitation.

The isothiocyanate group of DTTA reacts with primary amines in proteins and other molecules to form stable thiourea bonds (Figure 10.2) (Mukkala *et al.*, 1989). The reagent is water-soluble and can be reacted under relatively mild conditions (in 0.1 M sodium carbonate, pH 9.0). The isothiocyanate group is reasonably stable in aqueous solution for short periods, but will degrade. Best results will be obtained if fresh DTTA is used. The reaction involves attack of the nucleophile on the central, electrophilic carbon of the isothiocyanate group. The resulting electron shift creates a thiourea linkage between the chelating compound and the protein with no leaving group. Modification of antibodies can be done without loss of significant antigen-binding activity, even when up to 10–15 DTTA chelates are substituted per immunoglobulin molecule (Stahlberg *et al.*, 1993). Diagnostic assays using DTTA–Eu^{3+} chelates are commercially available employing the DELFIA® (Wallac Oy, Turku, Finland) time-resolved fluoroimmunoassay system.

4. DFA

DFA or deferoxamine is *N′*-[5-[[4-[[5-(acetylhydroxyamino)pentyl]amino]-1,4-dioxobutyl]-hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide, a naturally occurring product

isolated from *Streptomyces pilosus*. Its native activity is forming iron complexes, but it is also very proficient at forming coordination chelates with other metals, particularly galium-68 (Motta-Hennessy *et al*., 1985). Radiopharmaceutical agents for imaging can be produced by modification of targeting molecules with DFA complexes containing radioactive metals. The amine group of DFA can be utilized for direct labeling of antibodies and other proteins through coupling with available carboxylates using carbodiimide-mediated conjugation with EDC (Chapter 3, Section 1.1). The use of amine-reactive, homobifunctional crosslinkers (Chapter 4) also can be employed to modify proteins with DFA at their amino groups.



DFA
Deferoxamine
MW 560.71

DFA–polymer conjugates can be made containing multiple chelating groups along the length of the polymer. The polymer backbone utilized for this synthesis can be either activated dextran (Torchilin *et al*., 1989) or succinylated poly-L-lysine (Slinkin *et al*., 1990; Torchilin *et al*., 1993). These DFA–polymer constructs can be attached to antibody molecules through additional functional groups on the polymer. Chelating polymers can provide much higher signals than direct attachment of DFA to antibodies, since each coupled polymer derivative can possess dozens of chelated radioactive metals. In addition, high substitution levels of DFA directly coupled to antibodies can significantly affect activity by denaturation or blocking of the antigen-binding sites.

5. Use of Thiolation Reagents for Direct Labeling to Sulfhydryl Groups

Proteins containing sulfhydryl residues can be labeled with a radioactive element by direct complexation to the —SH group through a dative bond (Chapter 2, Section 2.8), avoiding entirely the use of a BCA. Particularly, reduced sulfhydryls in antibody molecules can be coupled with ^{99m}Tc to yield thiol-metal derivatives (Rhodes, 1991; Thakur and DeFulvio, 1991). However, cleavage of disulfide linkages within the antibody can lead to activity losses and fragmentation (Pimm *et al*., 1991). The required sulfhydryl groups can be introduced into antibodies without disulfide reduction through the use of a thiolating reagent that modifies amine residues within the antibody (Joiris *et al*., 1991). Thiolating agents such as 2-iminothiolane or SATA

(*N*-Succinimidyl-*S*-acetylthioacetate) provide efficient ways of introducing multiple sulfhydryl groups for this type of radiopharmaceutical preparation (Chapter 1, Section 4.1).

Site-directed thiolation at carbohydrate residues within the Fc region of antibody molecules may prove to be the best choice for —SH group introduction while maintain antigen-binding activity. Ranadive *et al.* (1993) have used the heterobifunctional crosslinking agent PDPH (3-(2-pyridyldithio)propionyl hydrazide) (Chapter 5, Section 2.3) to react specifically with oxidized polysaccharide components of monoclonals. The polysaccharide chains are treated first with sodium periodate (Chapter 1, Section 4.4) to generate reactive aldehyde residues. PDPH then is coupled to these aldehydes via its hydrazide end to create stable hydrazone linkages. The other end of the crosslinker, containing a pyridyl disulfide group, is reduced with DTT under mild conditions (25 mM DTT, pH 4.5, 30 minutes) to produce the free sulfhydryl groups. Since the thiolation occurs only at carbohydrate locations within the antibody, the modification has a better chance of being away from the antigen-binding sites, thus preserving immunoglobulin activity. Subsequent treatment with sodium pertechnatate yields the ^{99m}Tc derivative on the sulfhydryl groups (Figure 10.3).

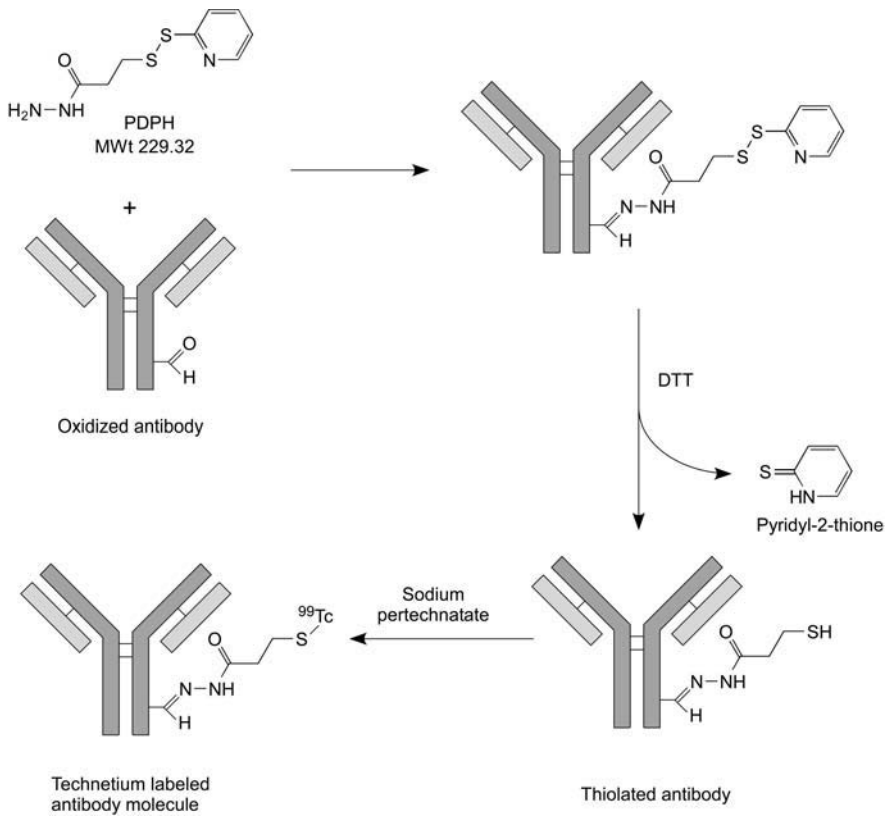
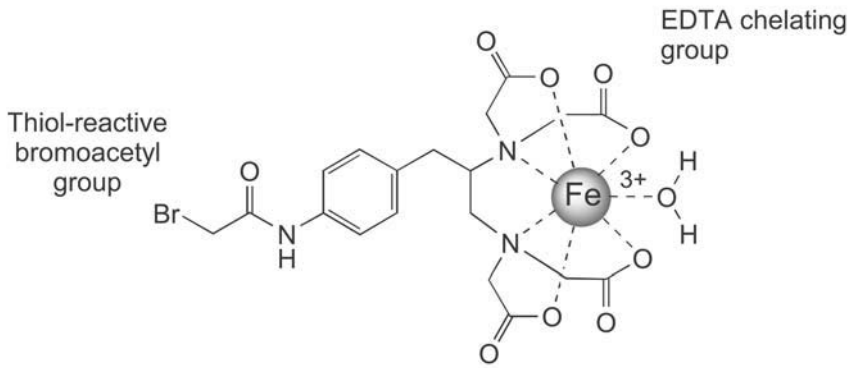


Figure 10.3 Antibody molecules oxidized with sodium periodate to create aldehyde groups on their polysaccharide chains can be modified with PDPH to produce thiols after reduction of the pyridyl disulfide. Direct labeling of the sulfhydryls with ^{99}Tc produces a radioactive complex.

6. FeBABE

FeBABE (pronounced "iron-babe") is Fe(III) (S)-1-(*p*-bromoacetamido-benzyl)ethylene diamine tetraacetic acid, a BCA designed to a hydroxyl radical probe to footprint interacting domains between proteins. The bromoacetyl group reacts with thiols to form a thioether bond. The EDTA chelating group coordinates Fe^{3+} to create a redox active complex that is able to form reactive oxygen species in aqueous solution. Iron-EDTA chelates are effective at generating hydroxyl radicals (•OH) that can react with peptide bonds to oxidize and cut them non-selectively. The reaction is initiated with the addition of peroxide and ascorbate`, which catalyzes the peptide cleavage reaction. Thus, one protein labeled with FeBABE can be allowed to interact with a protein binding partner and the cleavage reaction initiated, which results in cuts in the peptide structure immediately surrounding the region where FeBABE is attached. The pattern of peptide cutting can be used to determine the area on both interacting proteins that constitutes the binding region, because all other peptide regions will be affected by the oxidation reaction.



Fe-BABE
Fe(III) (S)-1-(*p*-bromoacetamido-benzyl) ethylene diamine tetraacetic acid
MW 589.15

Chapter 28, Section 4 provides additional information about the FeBABE chelator and its use in studying protein interactions.

11

Biotinylation Reagents

The highly specific interaction of (strept)avidin with the small vitamin biotin can be a useful tool in designing assay, detection, and targeting systems for biological analytes (see Chapter 23). The extraordinary affinity of (strept)avidin's interaction with biotin allows biotin-containing molecules in complex mixtures to be discretely bound with (strept)avidin conjugates. If the (strept)avidin–biotin complex contains detection components, then the targeted analytes can be located or quantified. This assay concept is made possible through the ability of biotin to be covalently attached to other targeting molecules, such as antibodies. In this sense, biotin derivatives may be prepared which contain reactive portions able to couple with particular functional groups in proteins and other molecules. Biotin modification of secondary molecules, called *biotinylation*, results in covalent derivatives containing one or more bicyclic biotin rings extending from the parent structure. These biotinylation sites are still capable of binding avidin or streptavidin with the specificity and nearly the same avidity of free biotin in solution. Since the biotin components are relatively small, macromolecules can be modified with these reagents without significantly affecting their physical or chemical properties (Della-Penna *et al.*, 1986).

The basic design of a biotin labeling compound is illustrated in Figure 11.1. Common to all such modification reagents is the presence of the bicyclic biotin ring at one end of the structure and a reactive group at the other end that can be used to couple with other molecules. Biotinylation reagents also possess various cross-bridges or spacer groups built off the valeric acid side chain of the molecule. Since the binding sites for biotin on avidin and streptavidin are pockets buried about 9 Å beneath the surface of the proteins, spacers can affect the accessibility of biotinylated compounds for efficiently binding avidin or streptavidin conjugates (Green *et al.*, 1971). In some applications, the use of a long spacer arm in the biotinylation reagent will result in the greatest potential assay sensitivity. The rate of binding of an avidin or streptavidin probe to a biotinylated molecule also is affected by the length of spacer in the biotinylation reagent. When longer spacers are utilized to make biotinylated macromolecules, it potentially can result in a 5-fold greater rate of streptavidin interaction (Bonnard *et al.*, 1984).

Another variable to consider in choosing biotinylation reagents is the use of a biotin analog such as iminobiotin that has a moderated affinity constant in its binding of avidin or streptavidin (Section 3.1, this chapter). Analogs may be useful if release of the (strept)avidin–biotin bond is important for isolating a targeted analyte. Using native biotin, the interaction with avidin is so strong that up to 6–8 M guanidine at pH 1.5 is required to break the bond, possibly

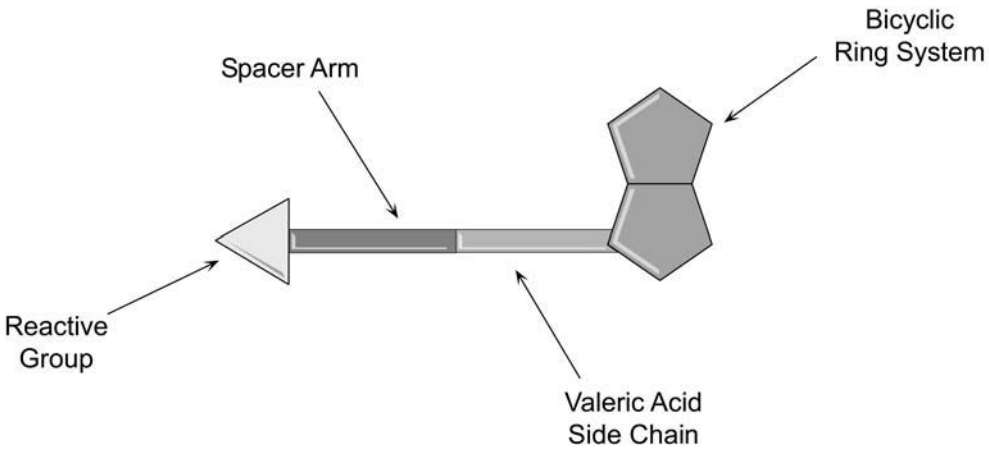


Figure 11.1 The basic design of a biotinylation reagent includes the bicyclic rings and valeric acid side chain of D-biotin at one end and a reactive group to couple with target groups at the other end. Spacer groups may be included in the design to extend the biotin group away from modified molecules, thus ensuring better interaction capability with avidin or streptavidin probes.

causing extensive denaturation of any other complexed molecules. By contrast, iminobiotinylated molecules can be released simply by adjusting the pH down to 4.

The following sections discuss some of the more common biotinylation reagents available for modification of proteins and other biomolecules. Each biotin derivative contains a reactive portion (or can be made to contain a reactive group) that is specific for coupling to a particular functional group on another molecule. Careful choice of the correct biotinylation reagent can result in directed modification away from active centers or binding sites, and thus preserve the activity of the modified molecule.

Additional biotinylation reagents are discussed in Chapter 18, Section 3, which describes newer hydrophilic biotin compounds containing PEG spacers. These reagents offer significant advantages over the more traditional aliphatic compounds, because the pronounced water-solubility of the PEG cross-bridge can prevent aggregation of biotinylated molecules. With some longer chain biotin compounds that contain hydrophobic hydrocarbon spacers, proteins can precipitate or lose activity over time due to the insolubility of the biotin modifications. Biotinylated antibodies are particularly susceptible to aggregation and loss of antigen binding ability if they are modified using hydrophobic biotin compounds. The PEG-based biotin reagents show better solubility and longer stability than their corresponding aliphatic biotinylation compounds of equivalent size.

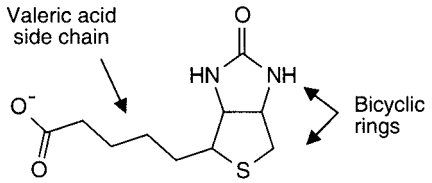
1. Amine-Reactive Biotinylation Agents

Amine-reactive biotinylation reagents contain reactive groups off biotin's valeric acid side chain that are able to form covalent bonds with primary amines in proteins and other molecules. Two basic types are commonly available: *N*-hydroxysuccinimide (NHS) esters and carboxylates. NHS esters spontaneously react with amines to form amide linkages (Chapter 2, Section 1.4),

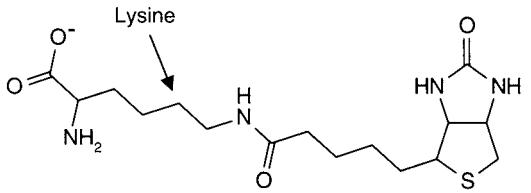
whereas carboxylate-containing biotin compounds can be coupled to amines via a carbodiimide-mediated reaction using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (Chapter 3, Section 1.1).

1.1. D-Biotin and Biocytin

D-Biotin (hexahydro-2-oxo-1H-thieno[3,4-d]imidazole-4-pentanoic acid) is a naturally occurring growth factor present in small amounts within every cell. It is a key component in numerous processes involving carboxylation reactions, wherein it functions as a cofactor and transporter of CO_2 (coenzyme R). Biotin is mainly found covalently attached to lysine ε-amine groups of proteins via its valeric acid side chain. The compound was originally discovered through symptoms of deficiency caused by eating too many raw egg-whites. Biotin (or vitamin H) was found to be complexed and inactivated by the egg-white protein avidin (Boas, 1927; du Vigneaud, 1940). Treatment with additional vitamin H alleviated the symptoms.



D-Biotin
MW 244.31



Biocytin
MW 372.48

The interaction of biotin with the proteins avidin and streptavidin is among the strongest noncovalent affinities known ($Ka = 10^{15}\ M^{-1}$). The binding occurs between the bicyclic ring of biotin and a pocket within each of the 4 subunits of the proteins. The valeric acid portion is not directly involved with the interaction with avidin (Green, 1975; Wilchek and Bayer, 1988), but elimination of its carboxylate group or changing the amide linkage in biotinylation compounds can affect its affinity for (strept)avidin. Amide derivatives of biotin's valeric acid group, however, can be made without interfering with its high affinity interactions. This characteristic allows modification of the valeric acid side chain without affecting the binding potential toward avidin or streptavidin.

D-Biotin is thus the basic building block for constructing biotinylation reagents. The molecule may be attached directly to a protein via its valeric acid side chain or derivatized at this carboxylate with other organic components to create spacer arms and various reactive groups. Reaction of biotin with primary amine groups on proteins can be done using the water-soluble carbodiimide, EDC (Chapter 3, Section 1.1). EDC activates the carboxylate to create a highly reactive, intermediate ester. This ester then can couple to amines to form stable amide bond derivatives (Figure 11.2). Biotinylated molecules thus formed retain the ability to bind avidin or streptavidin with high affinity.

The only potential deficiency in using D-biotin to modify directly a protein is the relatively short spacer arm afforded by the indigenous valeric acid group. Some applications may require longer spacers to maintain good binding potential toward avidin or streptavidin.

Biocytin is ε-*N*-biotinyl-L-lysine, a derivative of D-biotin containing a lysine group coupled at its ε-amino side chain to the valeric acid carboxylate. It is a naturally occurring complex of biotin that is typically found in serum and urine, and probably represents breakdown products of recycling biotinylated proteins. The enzyme biotinidase specifically cleaves the lysine residue and releases the biotin component from biocytin (Ebrahim and Dakshinamurti, 1986, 1987).

Biocytin has been used extensively as a labeling reagent for intracellular components within neurons (Horikawa and Armstrong, 1988; King *et al.*, 1989; Izzo, 1991; Granata and Kitai, 1992). It is particularly good for anterograde tracing studies in the central nervous system, since it can be easily injected into neurons using micropipettes. Subsequent visualization of biocytin locations may be done using an (strept)avidin–enzyme conjugate (Chapter 23, Section 3).

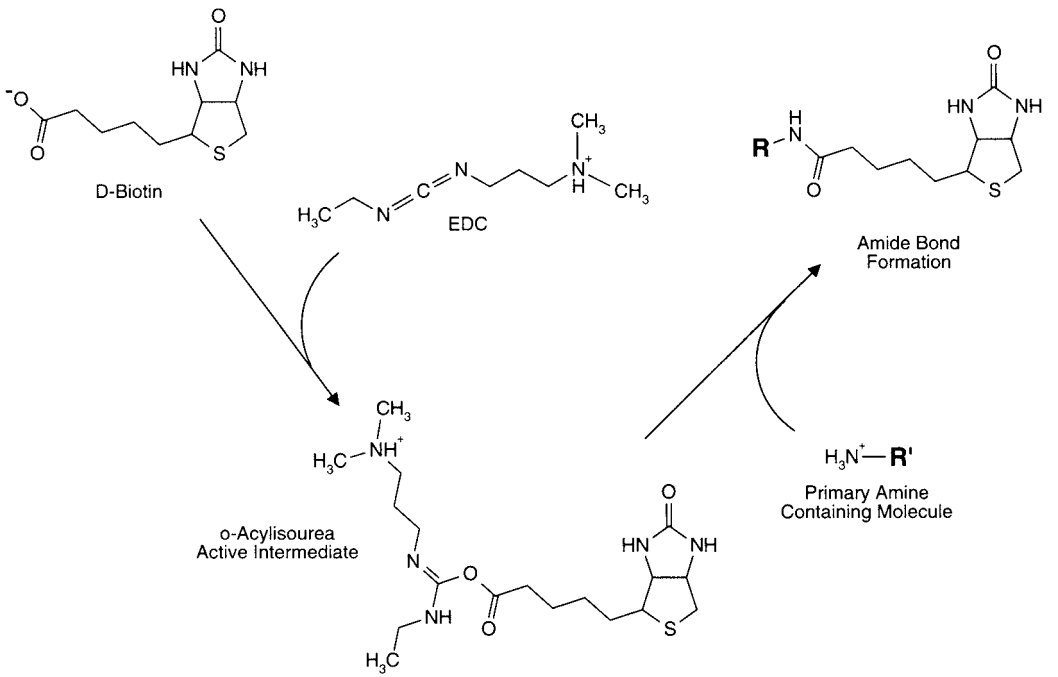


Figure 11.2 D-Biotin can be directly coupled to amine-containing molecules using the water-soluble carbodiimide EDC to form an amide bond linkage.

Biocytin should not be used in a carbodiimide reaction to modify proteins or other molecules, since it contains both a carboxylate and an amine group. A carbodiimide-mediated reaction, as suggested for D-biotin previously, would cause self-conjugation and polymerization of this reagent.

Biocytin, however, can form the basis for constructing trifunctional crosslinking reagents (Chapter 6). The lysine component of the molecule contains a free carboxylate and an α-amine group that can be used to build spacers and reactive groups for crosslinking purposes. The biotin component is the third arm of the trifunctional system, retaining its ability to bind (strept)avidin probes after conjugation has occurred at its other two ends. Such a trifunctional derivative has been used to study the hormone binding site of the insulin receptor (Wedekind *et al.*, 1989). This compound, 4-azido-2-nitrophenyl-biocytin-4-nitrophenyl ester, contains an amine-reactive group and a photoreactive phenyl azide functionality (Chapter 6, Section 1). The nitrophenyl ester reacts with amines on proteins and other molecules to from stable amide linkages. Once a molecule is modified in this manner, it contains both a photosensitive group and a biotin handle for conjugation and detection, respectively. Interaction of the modified protein with another protein and subsequent photolysis with UV light results in covalent crosslinking. Localization and detection of the crosslinked molecules then can be done using an avidin or streptavidin conjugate. Another trifunctional compound, sulfo-SBED (or sulfosuccinimidyl-2-[6-(biotinamido)-2-(pazidobenzamido) hexanoamido] ethyl-1,3′-dithiopropionate), also is based on a biocytin core (Chapter 6, Section 2). Additional information on use of these trifunctional biotin compounds based upon biocytin is described in Chapter 28, related to the study of protein interactions.

1.2. NHS-Biotin and Sulfo-NHS-Biotin

The valeric acid carboxylate of D-biotin may be activated to an NHS ester for direct modification of amine groups in proteins and other molecules. NHS esters react by nucleophilic attack of an amine on the carbonyl group, releasing the NHS group, and forming a stable amide linkage (Chapter 2, Section 1.4) (Figure 11.3). NHS-biotin is the simplest biotinylation reagent

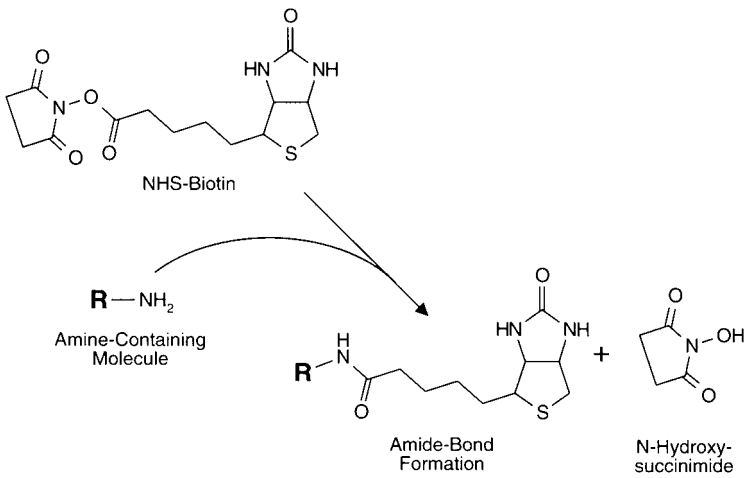
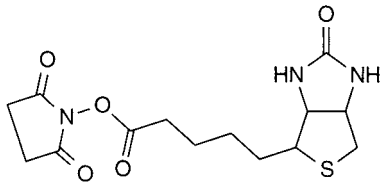
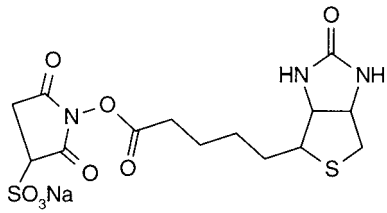


Figure 11.3 The active ester group of NHS-biotin reacts with amine-containing compounds to form amide bond linkages.

available. Modification reactions are carried out under mildly alkaline conditions, and they usually result in a high efficiency of biotin incorporation.



NHS-Biotin
MW 341.38
13.5 Å



Sulfo-NHS-Biotin
MW 443.42
13.5 Å

NHS-biotin is insoluble in aqueous environments. It must be dissolved first in organic solvent as a concentrated stock solution and an aliquot added to an aqueous reaction medium to facilitate dissolution. Organic solvents such as dimethylformamide (DMF) or Dimethyl sulfoxide (DMSO) are suitable for this purpose. Addition of an NHS-biotin solution to a reaction should not exceed a level of about 10 percent organic solvent in the buffer to avoid protein precipitation problems. Once added to the reaction medium, NHS-biotin may appear as a cloudy or hazy suspension, indicating incomplete solubility. However, such micro-dispersions still are effective at modification, often driving the bulk of the reagent into solution as the NHS ester reacts. Biotinylation of peptides or other molecules that are water-insoluble may be done completely in organic solvent. For example, insulin can be biotinylated with NHS-biotin in an organic medium (Hofmann *et al.*, 1977).

A water-soluble analog of NHS-biotin containing a negatively charged sulfonate group on its NHS ring structure also is available. Sulfo-NHS-biotin may be added directly to aqueous reactions without the need for organic solvent dissolution. A concentrated stock solution may be prepared in water to facilitate the addition of a small quantity to a reaction, but hydrolysis of the NHS ester will occur at a rapid rate, so the solution must be used immediately.

The only disadvantage to the use of NHS-biotin or sulfo-NHS-biotin is the lack of a long spacer group off the valeric acid side chain. Since the binding site for biotin on avidin and streptavidin is somewhat below the surface of the proteins, some biotinylated molecules may not interact as efficiently with (strept)avidin as when longer cross-bridges are used (Green *et al.*, 1971; Bonnard *et al.*, 1984).

NHS esters of D-biotin have been used in many applications, including the biotinylation of rat IgE to study receptors on murine lymphocytes (Lee and Conrad, 1984), in the development of

an immunochemical assay for a post-synaptic protein and its receptor (LaRochelle and Froehner, 1986a), in the study of plasma membrane domains by biotinylation of cell-surface proteins in *Dictyostelium disoideum* amoebas (Ingalls *et al*., 1986), and for the detection of blotted proteins on nitrocellulose membranes after transfer from polyacrylamide electrophoresis gels (LaRochelle and Froehner, 1986b).

The following protocol is a generalized method for the biotinylation of a protein using sulfo-NHS-biotin.

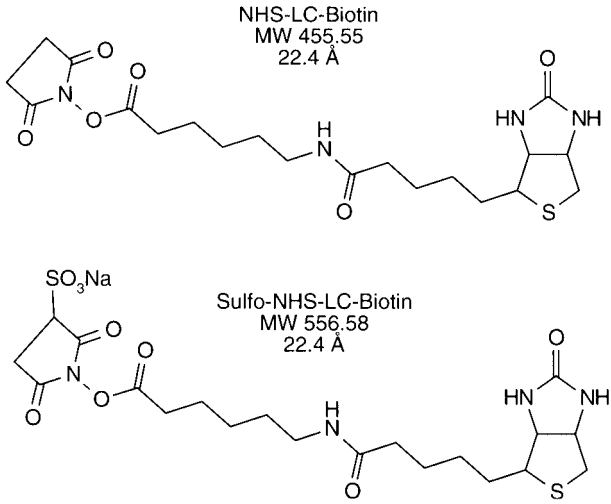
Protocol

1. Dissolve the protein to be biotinylated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5, at a concentration of 1–10 mg/ml.
2. Immediately before use, dissolve sulfo-NHS-biotin (Thermo Fisher) in water at a concentration of 20 mg/ml. Alternatively, the compound may be dissolved in organic solvent to prevent hydrolysis prior to a reaction (i.e., dry DMF or DMSO). Adjust the concentration and quantity of this stock solution to be prepared according to the amount of reagent needed to biotinylate the desired amount of protein. If prepared in water, the sulfo-NHS-biotin stock solution must be used immediately, since the NHS ester is subject to hydrolysis in aqueous environments.
3. With mixing, add a quantity of the sulfo-NHS-biotin solution to the protein solution to obtain a 12- to 20-fold molar excess of biotinylation reagent over the quantity of protein present. For instance, for an immunoglobulin (MW 150,000) at a concentration of 10 mg/ml, 20 µl of a sulfo-NHS-biotin solution (containing 8×10^{-4} mmol) should be added per ml of antibody solution to obtain a 12-fold molar excess. For more dilute protein solutions (i.e., 1–2 mg/ml), increased amounts of biotinylation reagent may be required (i.e., 20-fold molar excess or more) to obtain similar incorporation yields as when using more concentrated protein solutions.
4. React for 30–60 minutes at room temperature.
5. Purify the biotinylated protein from excess reagent and reaction by-products by gel filtration using a desalting resin or by dialysis against PBS.

Determination of the degree of biotinylation can be done using the HABA assay (Chapter 23, Section 7).

1.3. NHS-LC-Biotin and Sulfo-NHS-LC-Biotin

NHS-LC-biotin is a derivative of D-biotin containing a spacer arm off the valeric acid side chain, terminating in an NHS ester. The compound also is known as succinimidyl-6-(biotinamido)hexanoate or NHS-X-biotin. The 6-aminocaproic acid spacer provides greater length between a covalently modified molecule and the bicyclic biotin rings. The total distance from an attached molecule to the biotin component is about 22.4 Å, significantly greater than the 13.5 Å length of NHS-biotin without a spacer arm. This increased distance can result in better binding potential for avidin or streptavidin probes, because the binding sites on these proteins are buried relatively deep inside the surface plane.



The NHS ester end of NHS-LC-biotin reacts with amine groups in proteins and other molecules to form stable amide bond derivatives (Figure 11.4). Optimal reaction conditions are at a pH of 7–9, but the higher the pH the greater will be the hydrolysis rate of the ester. Avoid amine-containing buffers which will compete in the acylation reaction. NHS-LC-biotin is insoluble in aqueous reaction conditions and must be solubilized in organic solvent prior to the addition of a small quantity to a buffered reaction. Preparation of concentrated stock solutions may be done in DMF or DMSO. Nonaqueous reactions also may be done with this reagent for the modification of molecules insoluble in water. The molar ratio of NHS-LC-biotin to a

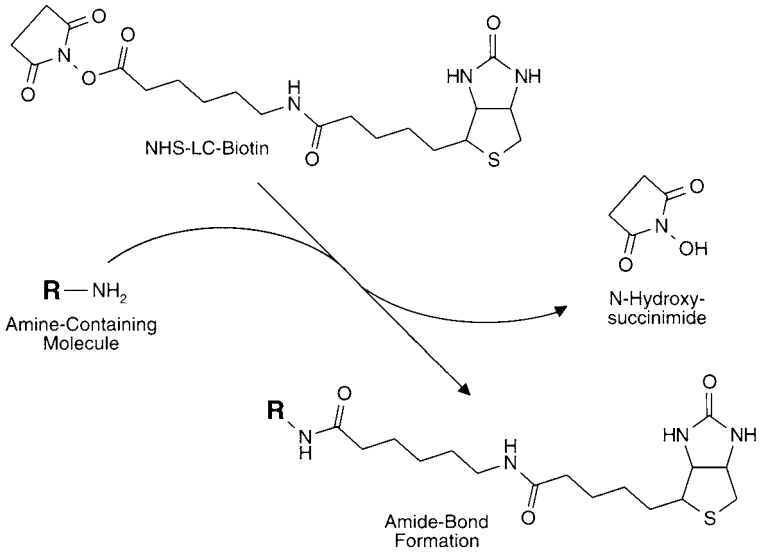


Figure 11.4 NHS-LC-biotin provides an extended spacer arm to allow greater distance between the biotin rings and a modified molecule. Reaction with amines forms amide linkages.

protein in a reaction can be from about 2:1 to about 50:1, with higher levels resulting in better incorporation yields (Gretch *et al*., 1987).

In a study comparing NHS-LC-biotin with two other derivatives of biotin, NHS-SS-biotin (Section 1, this chapter) and biotin hydrazide (Section 3, this chapter), it was found that modification through amines on monoclonal antibodies resulted in 2.5 times more activity in binding a streptavidinagarose affinity column than when modification of carbohydrate residues using hydrazide conjugation chemistry was done (Gretch *et al*., 1987). This was probably due to the greater abundance of amino groups over polysaccharide residues on these antibodies.

NHS-LC-biotin can be used to add a biotin tag to monoclonal antibodies directed at certain tumor antigens. The biotinylated monoclonals are allowed to bind to the tumor cell surfaces *in vivo*, and subsequent administration of an avidin or streptavidin conjugate can form the basis for inducing cytotoxic effects or creating traceable complexes for use in imaging techniques (Hnatowich *et al*., 1987).

The reagent also has been used in a unique tRNA-mediated method of labeling proteins with biotin for nonradioactive detection of cell-free translation products (Kurzchalia *et al*., 1988), in creating one- and two-step noncompetitive avidin–biotin immunoassays (Vilja, 1991), for immobilizing streptavidin onto solid surfaces using biotinylated carriers with subsequent use in a protein avidin–biotin capture system (Suter and Butler, 1986), and for the detection of DNA on nitrocellulose blots (Leary *et al*., 1983).

Sulfo-NHS-LC-biotin, a water-soluble analog of NHS-LC-biotin, also is available (Thermo Fisher) which contains a negatively charged sulfonate group on its NHS ring structure. The presence of the negative charge creates enough polarity within the molecule to allow direct solubility in aqueous reaction mediums. All other properties of the sulfonated version of the reagent are the same as those of NHS-LC-biotin.

Although NHS-LC-biotin and sulfo-NHS-LC-biotin are very popular reagents for biotinylation, they both result in hydrophobic aliphatic biotin modifications on proteins and antibodies. Unfortunately, these groups have a tendency to aggregate in aqueous solution and may cause protein precipitation or loss of activity over time. For this reason, the use of more hydrophilic PEG-based biotin compounds of approximately the same spacer length may be a better alternative for maintaining water solubility of modified proteins (Chapter 18).

The following protocol is a suggested method for the biotinylation of proteins with either NHS-LC-biotin or sulfo-NHS-LC-biotin.

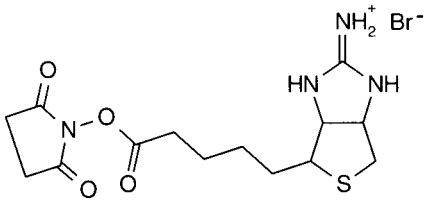
Protocol

1. Dissolve the protein to be biotinylated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5, at a concentration of 10 mg/ml.
2. Dissolve NHS-LC-biotin (Thermo Fisher) in dry DMF at a concentration of 40 mg/ml. This stock solution is stable for reasonable periods, although long-term storage is not recommended. For use of the water-soluble sulfo-NHS-LC-biotin, a stock solution may be prepared in either organic solvent or water, or the solid reagent may be added directly to the reaction mixture. If a solution in water is made to facilitate the addition of a small quantity of reagent to a reaction, then the solution should be prepared quickly and used immediately to prevent hydrolysis of the NHS ester. Sulfo-NHS-LC-biotin may be dissolved in water at a concentration of 20 mg/ml.

3. Add 50 µl of the NHS-LC-biotin solution in DMF to each ml of the protein solution in two aliquots apportioned 10 minutes apart. Alternatively, add a quantity of the sulfo-NHS-biotin solution prepared in water to the protein solution to obtain a 12- to 20-fold molar excess of biotinylation reagent over the quantity of protein present. For instance, for an immunoglobulin (MW 150,000) at a concentration of 10 mg/ml, 20 µl of the sulfo-NHS–biotin solution (8×10^{-4} mmol) should be added per ml of antibody solution to obtain a 12-fold molar excess.
4. React for a total of 30–60 minutes at room temperature or several hours at 4°C.
5. Remove unreacted biotinylation reagent and reaction by-products by gel filtration using a desalting resin or dialysis against PBS.
6. Assay for the level of biotin incorporation using the HABA dye procedure (Chapter 23, Section 7).

1.4. NHS-Iminobiotin

NHS-iminobiotin is *N*-hydroxysuccinimido-2-iminobiotin, the guanidino analog of NHS-biotin that has a lower affinity constant for binding avidin or streptavidin. Iminobiotin replaces the 2-oxo-imidazole upper ring structure of D-biotin with a 2-imino-imidazole structure, causing moderated interaction with the avidin or streptavidin binding sites. This biotin analog can be used in situations requiring mild dissociation of the (strept)avidin–biotin complex. Normally, breaking the (strept)avidin–biotin interaction requires 6–8 M guanidine hydrochloride at a pH of 1.5, an environment too severe for most proteins to maintain native structure or recover activity. Iminobiotin, by contrast, can be bound to avidin or streptavidin at a pH wherein the guanidino group is unprotonated and thus uncharged. Binding occurs at pH values above 9.5 (typically done with good affinity at pH 11), and elution can be accomplished simply by changing the pH to 4.0—an environment which protonates the 2-imino group and creates a positive charge—thus effectively dissociating the interaction (Figure 11.5).



NHS-Iminobiotin
N-Hydroxysuccinimido-
iminobiotin hydrobromide
MW 421.32
13.5 Å

NHS-iminobiotin can be used to label amine-containing molecules with an iminobiotin tag, providing reversible-binding potential with avidin or streptavidin. The NHS ester reacts with proteins and other amine-containing molecules to create stable amide bond derivatives (Figure 11.6). An iminobiotinylated molecule then can be used to target and purify other

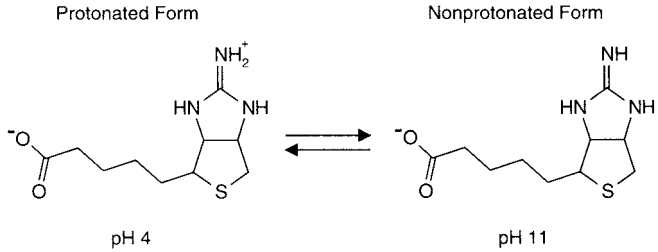


Figure 11.5 At pH 4, the protonated form of iminobiotin does not interact with the binding sites on avidin or streptavidin. At pH 11, the imino group is unprotonated and regains binding capability toward these proteins.

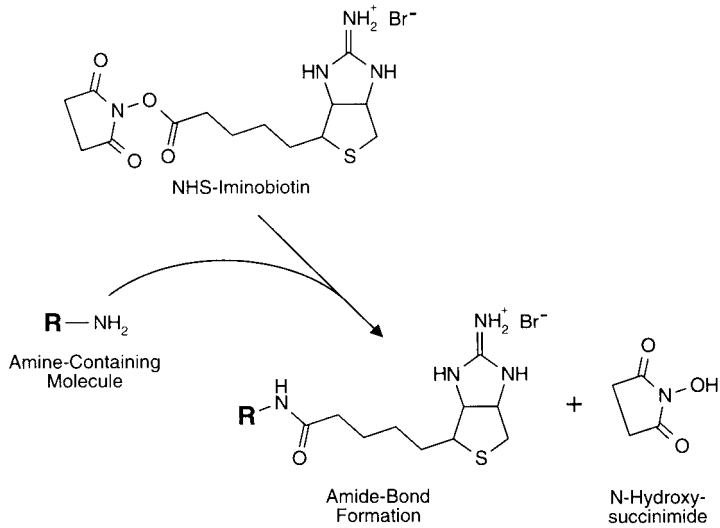


Figure 11.6 NHS-iminobiotin can be used to label amine-containing molecules, creating amide linkages.

components in biological samples. For instance, a targeting molecule, such as an antibody, can be iminobiotinylated and allowed bind its target in complex mixtures (such as tissue sections, cell extracts, or homogenates). The antibody–antigen complex subsequently can be purified using an affinity column of immobilized avidin with binding at pH 10–11 and simple elution at pH 4 (Orr, 1981; Zeheb *et al.*, 1983). The relatively mild elution condition allows recovery of the bound antigen without exposure to severely denaturing conditions.

The iminobiotin–avidin interaction also can be utilized in the opposite approach. Immobilized iminobiotin affinity columns can be used to purify avidin- or streptavidin-containing complexes under mild elution conditions (Hofmann *et al.*, 1980).

NHS-iminobiotin is insoluble in aqueous solution. It can be dissolved in organic solvent (DMF) prior to addition of a small aliquot to a buffered reaction medium. Don't exceed 10 percent DMF in the reaction to avoid protein precipitation problems. Optimal conditions for protein derivatization include non-amine-containing buffers at a pH of 7–9. The following

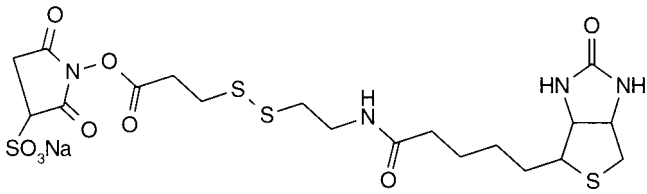
protocol is a suggested method for labeling antibodies with NHS-iminobiotin. Some optimization may have to be done for particular derivatization needs.

Protocol

1. Dissolve the antibody to be modified in 50 mM sodium borate, pH 8.0, at a concentration of 5 mg/ml.
2. Dissolve NHS-iminobiotin in DMF at a concentration of 1 mg/ml. Prepare fresh.
3. Add 100 µl of the NHS-iminobiotin solution to each ml of the antibody solution. Mix well to dissolve. *Note*: Some turbidity may be present in the reaction due to incomplete dissolution of the NHS-iminobiotin. The solution may look cloudy or have a microparticulate suspension present. This is normal for many water-insoluble reagents when added to an aqueous solution in an organic solvent. As the reaction takes place, the NHS-iminobiotin will be driven into solution, both by coupling to the protein and by hydrolysis of the NHS ester.
4. React for 30–60 minutes at room temperature or for 3 hours at 4°C.
5. Remove unreacted NHS-iminobiotin and reaction by-products by dialysis or gel filtration using a desalting resin.

1.5. Sulfo-NHS-SS-Biotin

Sulfo-NHS-SS-biotin (also known as NHS-SS-biotin) is sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate, a long-chain cleavable biotinylation reagent that can be used to modify amine-containing proteins and other molecules (Thermo Fisher). The cross-bridge of the compound provides a 24.3 Å spacer arm that creates plenty of distance between the modified molecule and the biotin end. Using a long-chain biotinylation reagent can increase the efficiency of biotinylated molecules to bind avidin or streptavidin conjugates, thus enhancing the potential sensitivity of assay systems.



Sulfo-NHS-SS-Biotin
Sulfosuccinimidyl-2-(biotinamido)-
ethyl-1,3-dithiopropionate
MW 606.7
24.3 Å

After molecules modified with sulfo-NHS-SS-biotin are allowed to interact with avidin or streptavidin probes, the complexes can be cleaved at the disulfide bridge by treatment with 50 mM DTT. Reduction releases the biotinylated molecule from the avidin or streptavidin capture reagent without breaking the (strept)avidin interaction. The use of disulfide biotinylation reagents

thus provides much gentler conditions to break the complex than would be required if the avidin–biotin interaction itself were disrupted (which dissociates only at 6–8 M guanidine, pH 1.5).

The use of a cleavable biotinylation reagent also provides a means to purify targeted molecules using affinity chromatography on a column of immobilized avidin or streptavidin. For instance, an antibody modified with sulfo-NHS-SS-biotin can be allowed bind its target in complex mixtures (such as tissue sections, cell extracts, or homogenates). The antibody–antigen complex subsequently can be isolated using an affinity column of immobilized avidin or streptavidin. Elution from the column with DTT breaks the disulfide bonds, releasing the antibody and its bound antigen. The isolation of Herpes virus proteins (Gretch *et al.*, 1987) and the recovery of DNA binding proteins (Shimkus *et al.*, 1985) were both done using this approach. Other methods of immunoprecipitation using non-cleavable biotinylation agents result in the inability to recover the captured proteins except under severely denaturing conditions.

Due to the presence of the negatively charged sulfonate group, sulfo-NHS-SS-biotin is a water-soluble biotinylation reagent that may be added directly to aqueous reactions without prior dissolution in organic solvent. For the addition of small quantities of reagent, the compound may be dissolved in water and an aliquot transferred to the reaction medium. If an aqueous stock solution of sulfo-NHS-SS-biotin is prepared, it must be dissolved rapidly and used immediately to prevent hydrolysis of the active ester. The NHS ester reaction forms stable amide linkages with amine-containing proteins and other molecules (Figure 11.7). Optimal conditions for the NHS ester reaction include a pH of 7–9, avoidance of any amine-containing buffers or other components that may compete in the reaction (including imidazole buffers

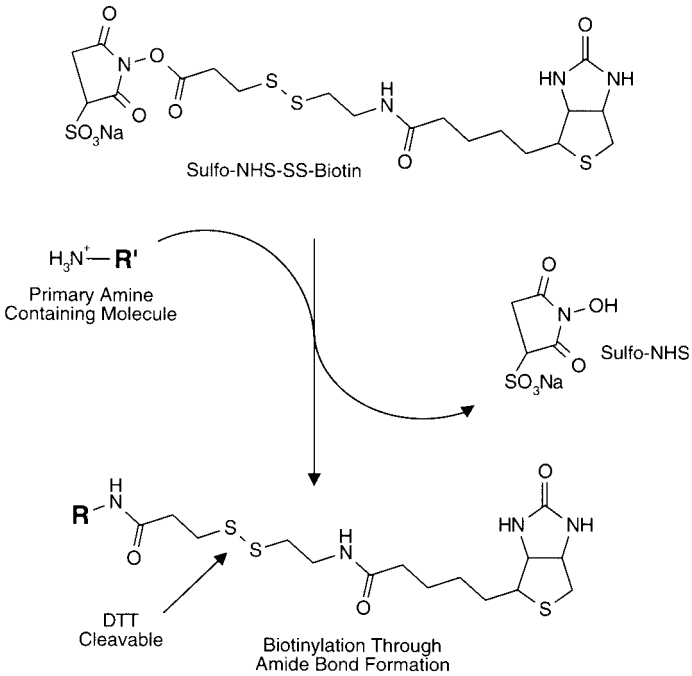


Figure 11.7 Sulfo-NHS-SS-biotin reacts with amine groups to form amide bonds. The biotin group can be later cleaved off the modified molecule by reduction of its internal disulfide linkage.

which catalyze hydrolysis of these esters), and avoidance of reducing agents that could cleave the disulfide bridge.

The following protocol is a suggested method for biotinylating antibody molecules with sulfo-NHS-SS-biotin. Some optimization may have to be done with each application to assure good biotin incorporation with retention of antigen binding activity. Other proteins and amine-containing molecules may be biotinylated using similar conditions.

Protocol

1. Dissolve the antibody to be biotinylated in 50 mM sodium bicarbonate, pH 8.5, at a concentration of 10 mg/ml. Other buffers and pH conditions between pH 7 and 9 can be used as long as no amine-containing buffers like Tris are present. Avoid also the presence of disulfide reducing agents that can cleave the disulfide group of the biotinylation reagent.
2. Add 0.3 mg of sulfo-NHS-SS-biotin (Thermo Fisher) to each ml of the antibody solution. To measure out small amounts of the biotinylation reagent, it may be first dissolved in water at a concentration of at least 1 mg/ml. Immediately transfer the appropriate amount to the antibody solution. This level of sulfo-NHS-SS-biotin addition represents about an 8-fold molar excess over the amount of antibody present. This should result in a molar incorporation of approximately 2–4 biotins per immunoglobulin molecule.
3. React for 30–60 minutes at room temperature or for 2–4 hours at 4°C.
4. Remove unreacted biotinylation reagent and reaction by-products by dialysis or gel filtration using a desalting resin.

Sulfo-NHS-SS-biotin also can be used to label cell-surface proteins for subsequent detection or isolation using (strept)avidin reagents. The negative charge character of the compound prior to its reaction with an amine on a protein prevents it from penetrating the cell membrane bilayer. Thus, proteins on the outer surface of the cell can be specifically tagged with a biotin group. The disulfide cross-bridge of sulfo-NHS-SS-biotin allows recovery of labeled proteins after capture on an immobilized (strept)avidin support. Reduction of the disulfide using DTT or TCEP releases the proteins without the severe denaturing conditions usually required to break the (strept)avidin–biotin interaction. This allows isolation of cell-surface proteins under nondenaturing conditions for subsequent analysis (Schuberth *et al.*, 1996; DeBlaquiere and Burgess, 1999; Ellerbroek *et al.*, 2001; Jang and Hanash, 2003).

The following protocol is based on the method of Thermo Fisher, as found in the instructions for the cell-surface biotinylation kit.

Protocol

1. Grow cells in four T75 cm^2 flasks until they are 90–95 percent confluent.
2. Remove the media and wash the cells twice with 8 ml of cold 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2 (PBS). *Note*: this buffer contains a high buffer salt content to stabilize the pH during the biotinylation reaction. Do not allow the cells to remain in contact with it for more than 5 seconds to prevent detachment from the flask surface.
3. Dissolve 12 mg of sulfo-NHS-SS-biotin in 48 ml of cold PBS and immediately add 10 ml of the solution to each flask containing the washed cells.
4. React with gentle rocking for 30 min at 4°C.
5. Quench the reaction by the addition of 1 ml of 1 M Tris, pH 7.2.

6. Scrape the cells from each flask and transfer them into a 50 ml tube. Wash each flask using a single 10 ml portion of 0.025 M Tris, 0.15 M NaCl, pH 7.2, and add the solution to the scraped cells.
7. The isolated cells may be lysed using standard mechanical or detergent methods and the biotinylated cell-surface proteins analyzed or isolated using (strept)avidin reagents.

2. Sulfhydryl-Reactive Biotinylation Agents

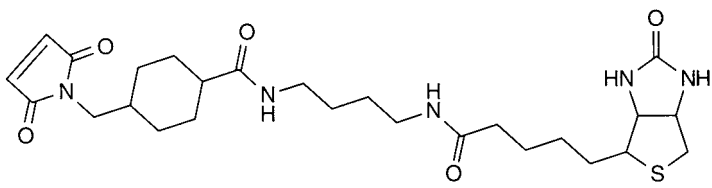
Sulfhydryl-reactive biotinylation reagents allow modification at cysteine —SH groups or at sites of specific thiolation within proteins and other molecules. Targeting sulfhydryls for modification, as opposed to amines, usually results in more limited derivatization, often away from active centers or binding sites. Directed coupling of biotin in this manner can aid in preserving activity. For instance, antibodies may be cleaved by reduction at their disulfide groups in the hinge region, forming free sulfhydryls removed from the antigen binding site (Chapter 20, Section 1.1). Biotinylation at these sites produces a derivative that can bind efficiently to both antigen and (strept)avidin probes without steric hindrance.

Sulfhydryl groups also can be added to 5′-phosphate end of DNA probes (Chapter 27, Section 2.2). Biotinylation at these sites avoids disruption of base pairing with complementary DNA targets, since the point of modification is restricted to a single end position on the oligonucleotide.

The following sections discuss three sulfhydryl-reactive biotinylation reagents that utilize maleimide, pyridyl disulfide, and iodoacetyl reactive groups, respectively. The maleimide and iodoacetyl options produce nonreversible, covalent thioether linkages with target —SH groups. The pyridyl disulfide chemistry results in disulfide bonds that are reversible through cleavage with a reducing agent.

2.1. Biotin-BMCC

Biotin-BMCC is 1-biotinamido-4-[4′-(maleimidomethyl)cyclohexane-carboxamido]butane, a biotinylation reagent containing a maleimide group at the end of an extended spacer arm (Thermo Fisher). The maleimide end reacts with sulfhydryl groups in proteins and other molecules to form stable thioether linkages (Figure 11.8). The reaction is highly specific for —SH groups in the range of pH 6.5–7.5. The long spacer arm (32.6 Å) provides more than enough distance between modified molecules and the bicyclic biotin end to allow efficient binding of avidin or streptavidin probes.



Biotin-BMCC
1-Biotinamido-4-[4'-(maleimidomethyl)-
cyclohexane-carboxamido]butane
MW 533.69
32.6 Å

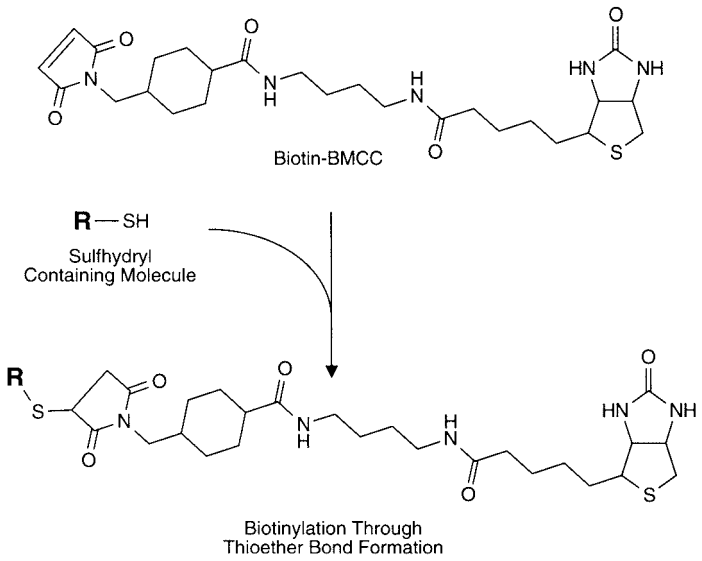


Figure 11.8 Biotin-BMCC provides sulfhydryl reactivity through its terminal maleimide group. The reaction creates a stable thioether linkage.

The reagent is similar to another maleimide-containing biotinylation reagent, 3-(*N*-maleimidopropionyl) biocytin, a compound used to detect sulfhydryl-containing molecules on nitrocellulose blots after SDS-electrophoresis separation (Bayer *et al.*, 1987). Biotin-BMCC should be useful in similar detection procedures.

Biotin-BMCC is insoluble in water and must be dissolved in an organic solvent prior to addition to an aqueous reaction mixture. Preparing a concentrated stock solution in DMF or DMSO allows transfer of a small aliquot to a buffer reaction. The upper limit of biotin-BMCC solubility in DMSO is approximately 33 mM or 17 mg/ml. In DMF, it is only soluble to a level of about 7 mM (4 mg/ml). Upon addition of an organic solution of the reagent to an aqueous environment (do not exceed 10 percent organic solvent in the aqueous medium to prevent protein precipitation), biotin-BMCC may form a micro-emulsion. This is normal and during the course of the reaction, the remainder of the compound will be driven into solution as it couples or hydrolyzes.

The required sulfhydryl groups for biotin-BMCC modification may be indigenous in molecules, formed through reduction of disulfides, or created by the use of thiolation reagents (Chapter 1, Section 4.1). At physiological pH, the rate of the maleimide reaction toward sulfhydryls is almost 1,000-fold faster than its reaction toward amines. However, at higher pH values the maleimide will couple to amines quite readily (Wu *et al.*, 1976; Ishi and Lehrer, 1986). Maleimides also can undergo a ring-opening hydrolysis reaction which increases in rate with pH, effectively inactivating the reactive group for thiol coupling.

The following protocol is a suggested method for modifying sulfhydryl-containing proteins with biotin-BMCC. Some optimization of biotinylation levels may have to be done for particular applications.

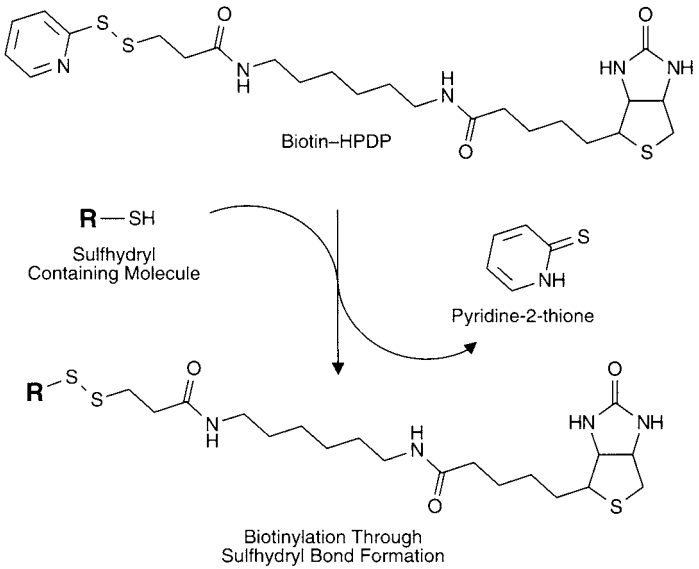


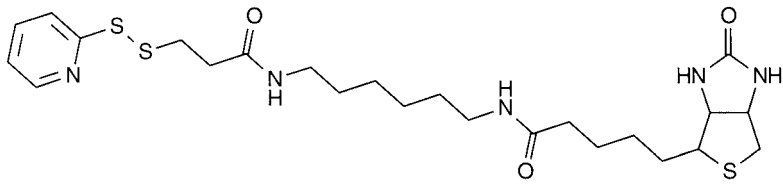
Figure 11.9 Biotin-HPDP reacts with sulfhydryl-containing molecules through its pyridyl disulfide group, forming reversible disulfide bonds. The biotin group may be released from modified molecules by reduction with DTT.

Protocol

1. Dissolve the protein to be biotinylated (containing one or more free sulfhydryls) in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 6.5–7.5, at a concentration of 2.5 mg/ml.
2. Dissolve biotin-BMCC (Thermo Fisher) in DMSO at a concentration of 5 mg/ml.
3. Add 100 µl of the biotin-BMCC solution to each ml of the protein solution. Mix well.
4. React for at least 2 hours at room temperature.
5. Remove excess biotinylation reagent and reaction by-products by dialysis or gel filtration using a desalting resin.

2.2. Biotin-HPDP

Biotin-HPDP is *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide (Thermo Fisher). The reagent contains a 1,6-diaminohexane spacer group which is attached to biotin's valeric acid side chain. The terminal amino group of the spacer is further modified via an amide linkage with the acid precursor of SPDP (Chapter 5, Section 1.1) to create a terminal, sulfhydryl-reactive group. The pyridyl disulfide end of biotin-HPDP can react with free thiol groups in proteins and other molecules to from a disulfide bond with loss of pyridine-2-thione (Figure 11.9). This leaving group may be monitored by its characteristic absorbance at 343 nm to assess the level of biotinylation. However, since its extinction coefficient is rather low (about $8 \times 10^3\ M^{-1}cm^{-1}$), small-scale biotinylations may not be quantifiable using this technique.



Biotin–HPDP
N-[6-(Biotinamido)hexyl]-3'-
(2'-pyridyldithio)propionamide
MW 539.77
29.2 Å

Modifications done with biotin-HPDP produce biotinylated compounds with long spacer arms (29.2 Å), assuring good binding efficiency with avidin or streptavidin probes. After coupling to sulfhydryl-containing molecules, the biotin-HPDP component can be cleaved by treatment with disulfide reducing agents, such as DTT. Breaking this bond releases the biotin modifications and regenerates the original sulfhydryl-containing molecule. This cleavability also provides a means of recovering target complexes after purification of the biotinylated molecules by affinity chromatography on immobilized avidin or streptavidin. Thus, biotin-HPDP-modified antibodies directed against some specific cellular antigen can be used to aid in the isolation of targeted components using affinity chromatography (immunoprecipitation) followed by elution with a disulfide reductant.

Using a similar approach, C1q has been modified with biotin-HPDP and allowed to interact with its specific receptor. Subsequent purification of the C1q receptor was accomplished through cleavage of the disulfide bridge of the biotinylation reagent (Ghebrehiwet *et al.*, 1988).

Biotin-HPDP is water-insoluble and therefore must be dissolved in an organic solvent prior to addition to an aqueous reaction medium. Suitable solvents include DMSO and DMF. Concentrated stock solutions may be prepared in DMSO and a small aliquot transferred to a buffered reaction solution. Do not add more than 10 percent organic solvent to the aqueous reaction to prevent precipitation or denaturation of biological molecules. After addition, a micro-emulsion may result. This is normal for many water-insoluble reagents. The solution usually will become clearer during the course of the reaction. Optimal conditions for the disulfide interchange reaction include a pH range of 6–9 in buffer systems that do not contain any extraneous sulfhydryl compounds or reducing agents such as DTT, 2-mercaptoethanol, or TCEP. If reducing agents are used to create sulfhydryls in the protein to be biotinylated, these must be completely removed by dialysis or gel filtration before reacting with biotin-HPDP.

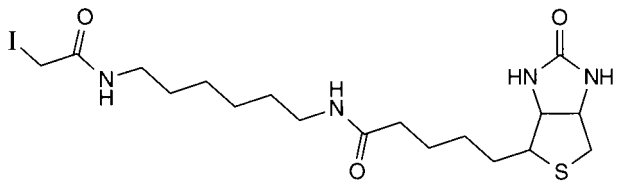
A suggested protocol for the use of biotin-HPDP in the modification of sulfhydryl-containing proteins follows. Similar procedures may be used when biotinylating other molecules.

Protocol

1. Dissolve the sulfhydryl-containing protein to be biotinylated in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2, at a concentration of at least 2 mg/ml.
2. Dissolve biotin-HPDP (Thermo Fisher) in DMSO at a concentration of 4 mM (2.1 mg/ml).
3. Add 100 µl of the biotin-HPDP stock solution to each ml of the protein solution. Mix well.
4. React for 90 minutes at room temperature.
5. Purify the biotinylated protein by gel filtration using a desalting resin or by dialysis. The PBS/EDTA buffer described in step 1 is suitable for either operation.

2.3. Iodoacetyl-LC-Biotin

Iodoacetyl-LC-biotin is *N*-iodoacetyl-*N*-biotinylhexylenediamine, a sulfhydryl-reactive biotinylation agent (Thermo Fisher). The reagent contains a 1,6-diaminohexane spacer group which is attached to biotin's valeric acid side chain. The terminal amino group of the spacer is further modified via an amide linkage with an iodoacetyl group to provide the sulfhydryl reactivity. Coupling to sulfhydryl-containing proteins or other molecules creates nonreversible thioether bonds (Figure 11.10). Modifications done with iodoacetyl-LC-biotin produce biotinylated compounds with sufficiently long spacer arms (27.1 Å) to assure excellent binding potential with avidin or streptavidin probes.



Iodoacetyl-LC-Biotin
N-Iodoacetyl-N-biotinylhexylenediamine
MW 510.42
27.1 Å

Iodoacetyl-LC-biotin is water-insoluble and therefore must be dissolved in an organic solvent prior to addition to an aqueous reaction medium. Suitable solvents include DMSO and DMF. Concentrated stock solutions may be prepared in DMSO and a small aliquot transferred

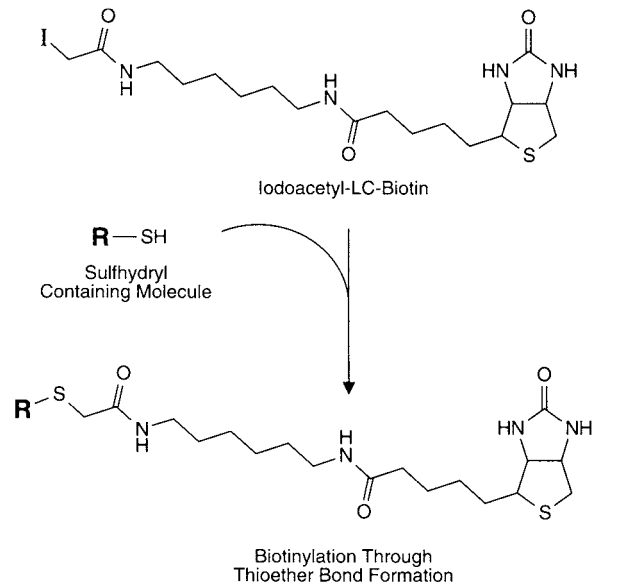


Figure 11.10 This biotinylation reagent reacts with sulfhydryl groups through its iodoacetamide end to form thioether bonds.

to a buffered reaction solution. Do not add more than 10 percent organic solvent to the aqueous reaction to prevent precipitation or denaturation of biological molecules. After addition, a microemulsion may result. This is normal for many water-insoluble reagents. The solution usually will become clear during the course of the reaction. Optimal conditions for coupling using iodoacetyl-containing reagents include a pH range of 7.5–8.5 in buffer systems that do not contain any extraneous sulfhydryl compounds. In addition, protect all solutions containing iodoacetyl-LC-biotin from light, since photolysis may cause liberation of iodine, degrading the activity of the compound and possibly causing modification of tyrosine or histidine residues by iodination.

Iodoacetyl-LC-biotin has been used to localize the SH_1 thiol of myosin by use of an avidin–biotin complex visualized by electron microscopy (Sutoh *et al.*, 1984) and to determine the spatial relationship between SH_1 and the actin binding site on the myosin subfragment-1 surface (Yamamoto *et al.*, 1984).

The following protocol is a suggested method for biotinylating sulfhydryl-containing proteins using iodoacetyl-LC-biotin. The required sulfhydryl groups may be provided through reductive cleavage of disulfide bonds or by the use of thiolation reagents (Chapter 1, Section 4.1). Other molecules may be modified with iodoacetyl-LC-biotin using similar techniques.

Protocol

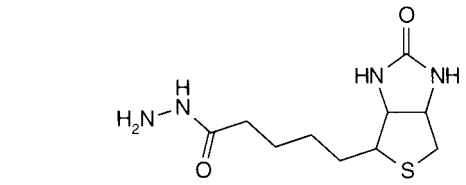
1. Dissolve the sulfhydryl-containing protein to be biotinylated in 50 mM Tris, 0.15 M NaCl, 10 mM EDTA, pH 8.3, at a concentration of 4 mg/ml.
2. Dissolve iodoacetyl-LC-biotin (Thermo Fisher) in DMF at a concentration of 4 mM (2 mg/ml). Protect from light.
3. Add 50 µl of the iodoacetyl-LC-biotin solution to each ml of the protein solution. Mix well. This level of addition represents a 3.28-fold molar excess of biotinylation reagent over the quantity of protein present if the protein has a molecular weight of 67,000 and possesses one sulfhydryl. Adjustments to the amount of reagent addition may have to be made to be appropriate for other proteins of different molecular weight. Consideration of the number of sulfhydryls present per protein molecule also should be done. React the biotinylation reagent at no more than a 3- to 5-fold molar excess over the amount of sulfhydryls present to assure specificity of the iodoacetyl group for only —SH groups. Higher ratios of reagent-to-protein may cause reaction with amine groups present on the protein.
4. React for 90 minutes in the dark at room temperature.
5. Remove excess reactants and reaction by-products by dialysis or gel filtration using a desalting resin.

3. Carbonyl- or Carboxyl-Reactive Biotinylation Agents

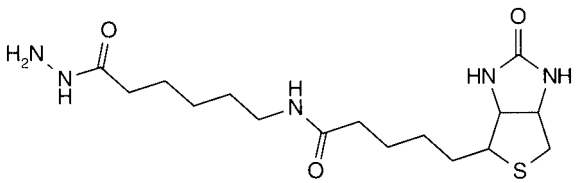
Hydrazide- or amine-containing biotinylation compounds can be used to modify carbonyl or carboxyl groups on other molecules. Hydrazides spontaneously react with aldehydes or ketones to give hydrazone linkages. The hydrazones may be further stabilized by reduction with sodium cyanoborohydride. The amine-containing biotinylation reagents (or the hydrazide ones) may be coupled to carboxylate groups using a carbodiimide reaction (Chapter 3, Section 1.1). In addition, amine- or hydrazide-containing biotinylation reagent may be coupled to cytosine residues in DNA or RNA by transamination catalyzed by bisulfite (Chapter 27, Section 2.3).

3.1. Biotin-Hydrazide and Biotin-LC-Hydrazide

Biotin-hydrazide is *cis*-tetrahydro-2-oxothieno[3,4-d]-imidazoline-4-valeric acid hydrazide, the hydrazine derivative of D-biotin off its valeric acid carboxylate (Thermo Fisher). The hydrazide functionality reacts with aldehyde and ketone groups to give hydrazone linkages. Although formyl groups are not common in biological molecules, they may be created by oxidation of diols with sodium periodate (Chapter 1, Section 4.4). Thus, glycoconjugates may be targeted specifically at their sugar residues. Biotinylation of these oxidized carbohydrates with biotin-hydrazide produces modifications which may be away from active centers or binding sites on proteins (Figure 11.11). Particularly, immunoglobulins may be biotinylated with this reagent at their polysaccharide groups which typically are present in the Fc region of the IgG molecule. Directed modification in this manner avoids the antigen binding sites at the ends of the heavy and light chains, thus preserving antibody activity and allowing avidin or streptavidin probes to dock without blocking or interfering with antigen binding (although care should be taken in this respect, as some antibodies contain carbohydrate near their antigen binding sites).



Biotin Hydrazide
MW 258.34
15.7 Å



Biotin-LC-Hydrazide
MW 371.5
24.7 Å

Biotin-hydrazide also may be used to couple with carboxylate-containing molecules. Hydrazides can be coupled with carboxylic acid groups by using the carbodiimide reaction (Chapter 3, Section 1.1). The carbodiimide activates a carboxylate to an *o*-acylisourea intermediate. Biotin-hydrazide can react with this intermediate via nucleophilic addition to form a stable covalent bond.

Biotin-hydrazide has been used to biotinylate antibodies at their oxidized carbohydrate residues (O'Shanessy *et al.*, 1984, 1987; O'Shanessy and Quarles, 1985; Hoffman and O'Shannessy, 1988), to modify the low-density lipoprotein (LDL) receptor (Wade *et al.*, 1985), to biotinylate nerve growth factor (NGF) (Rosenberg *et al.*, 1986), and to modify cytosine groups in oligonucleotides to produce probes suitable for hybridization assays (Reisfeld *et al.*, 1987) (Chapter 27, Section 2.3).

An analog of this biotinylation reagent with a longer spacer arm also exists. Biotin-LC-hydrazide contains a 6-aminocaproic acid extension off its valeric acid group (Thermo Fisher).

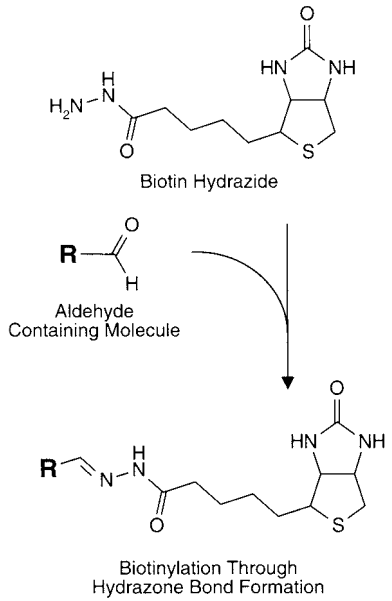


Figure 11.11 Biotin-hydrazide can be used to label aldehyde-containing molecules, creating hydrazone bonds.

The increased length of this spacer (24.7 Å) provides more efficient interaction potential with avidin or streptavidin probes, possibly increasing the sensitivity of assay systems. The reactions of biotin-LC-hydrazide are identical to those of biotin-hydrazide.

The following protocol describes the use of biotin-hydrazide to label glycosylated proteins at their carbohydrate residues. Control of the periodate oxidation level can result in specific labeling of sialic acid groups or general sugar residues (Chapter 1, Section 4.4).

Protocol

1. Dissolve a periodate-oxidized glycoprotein (i.e., antibodies—see Chapter 20, Section 1.3) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4, at a concentration of 2 mg/ml. *Note*: The buffer, 0.1 M sodium acetate, pH 5.5, is typical of literature references for reaction of a hydrazide compound with an aldehyde-containing molecule to form a hydrazone linkage. Alternative buffer conditions using higher pH values also work well. Physiological pH conditions with the use of a reducing agent such as sodium cyanoborohydride (step 4) produce the most efficient labeling yields when using hydrazide-containing reagents.
2. Add biotin-hydrazide or biotin-LC-hydrazide to a final concentration of 5 mM.
3. React for 2 hours at room temperature.
4. To reduce the hydrazone bonds to more stable linkages, cool the solution to 4°C and add an equal volume of 30 mM sodium cyanoborohydride in PBS. Incubate for 40 min. *Note*: If the presence of a reducing agent is detrimental to protein activity, eliminate this step. In most cases, the hydrazone linkage is stable enough for avidin–biotin detection experiments.
5. Remove excess reactants by dialysis or gel filtration using a desalting column.

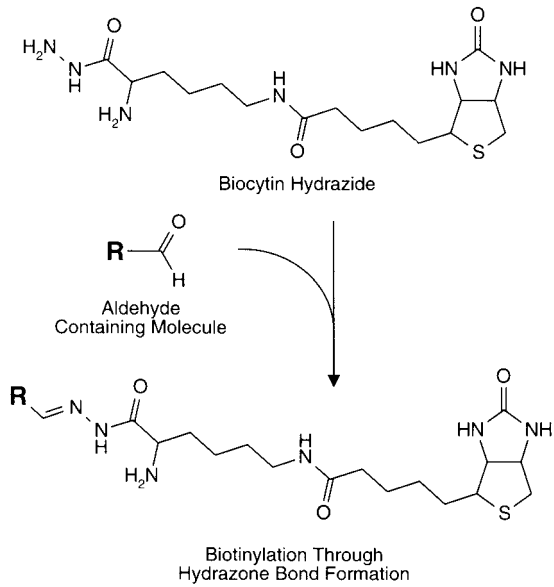
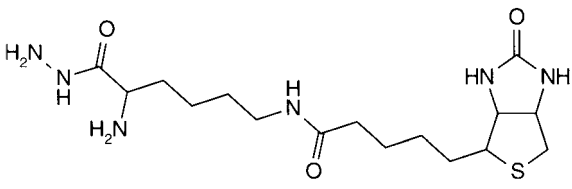


Figure 11.12 Biocytin-hydrazide reacts with aldehyde-containing molecules to form hydrazone bonds.

3.2. Biocytin Hydrazide

Another biotinylation reagent that can spontaneously couple with aldehyde- or ketone-containing molecules is biocytin hydrazide (Thermo Fisher). Produced by forming the hydrazine derivative of biocytina lysine–biotin complex often found naturally in serum (Section 1, this chapter)—the compound has better solubility in aqueous solutions than either biotin-hydrazide or biotin-LC-hydrazide discussed previously. The solubility enhancement of biocytin-hydrazide is due to the presence of lysine's α-amino group, which is protonated and positively charged at physiological pH. The reagent can be used to label carbohydrate-containing molecules, such as glycoproteins, after they have been oxidized to contain reactive aldehydes (Chapter 1, Section 4.4). The hydrazide group forms a hydrazone linkage with the aldehydes, thus directing the biotinylation reaction toward the polysaccharide regions of glycoconjugates (Figure 11.12).



Biocytin hydrazide was used to label specifically sialic acid residues, galactose residues, and for general sugar modification (Bayer *et al.*, 1988). The galactose residues were oxidized using galactose oxidase after treatment with neuraminidase (Chapter 1, Section 4.4). The use of this approach for labeling glycoproteins *in situ* was found to be optimal, due to the other potential side reactions that may occur when using sodium periodate.

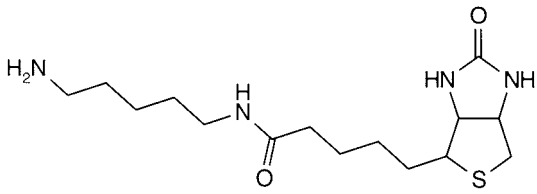
The reactivity and use of biocytin-hydrazide is similar to that described for biotin-hydrazide in Section 3, this chapter. The following protocol for labeling glycoproteins at oxidized carbohydrate (galactose) sites is from Bayer and Wilchek (1992).

Protocol

1. Dissolve the glycoprotein to be labeled in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (labeling buffer), at a concentration of 1 mg/ml.
2. Dissolve biocytin-hydrazide (Thermo Fisher) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS), at a concentration of 20 mg/ml.
3. To each ml of glycoprotein solution, add 30 µl of neuraminidase (1 unit/ml as supplied by Behringwerke AF), then 30 µl of galactose oxidase (previously dissolved at 100 units/ml in the labeling buffer of step 1), and finally 100 µl of the biocytin hydrazide solution.
4. React for 2 hours at 37°C.
5. Remove unreacted reagents by dialysis or gel filtration.

3.3. 5-(Biotinamido)pentylamine

The Derivative, 5-(biotinamido)pentylamine, contains a 5-carbon cadaverine spacer group attached to the valeric acid side chain of biotin (Thermo Fisher). The compound can be used in a carbodiimide reaction process to label carboxylate groups in proteins and other molecules, forming amide bond linkages (Chapter 3, Section 1). However, the main use of this biotinylation reagent is in the determination of factor XIIIa or transglutaminase enzymes in plasma, cell, or tissue extracts.



5-(Biotinamido)pentylamine
MW 328.48

Factor XIII, also known as plasma transglutaminase, is an enzyme of the blood coagulation cascade. It is activated by thrombin and calcium to factor XIIIa, at which point it catalyzes covalent crosslinks between the ε-amine group of lysine side chains and the γ-glutamyl side chain of glutamine residues. Abnormal levels of factor XIII in plasma are clinically important, being associated with cancer, liver or renal dysfunction, or various bleeding disorders. The assay of transglutaminase activity therefore is important for investigating the activity and function of this enzyme as it relates to post-translational protein modification as well as various disease states.

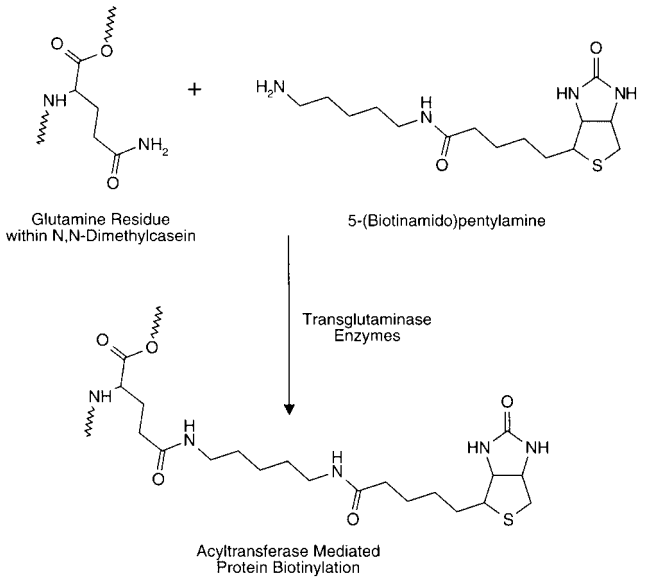


Figure 11.13 5-(Biotinamido)pentylamine can be used to label glutamine residues in proteins by enzymatic action of transglutaminase.

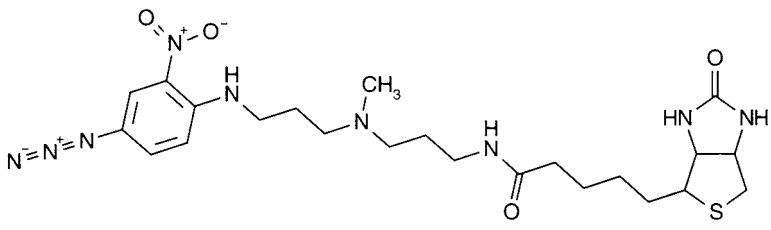
5-(Biotinamido)pentylamine is able to participate in the acyltransferase reaction, becoming covalently attached to protein substrates at their glutamine residues (Figure 11.13). Lee *et al.* (1988) used this biotinylation reagent to quantify factor XIII in plasma. Transglutaminase activity resulted in the modification of an *N,N′*-dimethylcasein substrate which was subsequently detected by an avidin–biotin assay procedure. The assay may be done in microplates using wells coated with the substrate protein and quantifying the enzyme activity with streptavidin-alkaline phosphatase (Slaughter *et al.*, 1992). Jeon *et al.* (1989) subsequently applied the assay to the measurement of transglutaminase activity in cells. Components biotinylated in cellular systems also can be isolated by use of affinity chromatography on immobilized avidin (Lee *et al.*, 1992).

4. Photoreactive Biotinylation Agents

Biotin derivatives containing a photoreactive group provide nonselective biotinylation potential at certain reactive hydrogen sites or nucleophilic groups. They can be used to incorporate an avidin binding, biotin group into molecules that do not possess amines, sulfhydryls, or other easily modifiable functional groups. Many of these photoreactive derivatives utilize the phenyl azide type of photosensitive group, which can be activated by exposure to UV light to an intermediate nitrene or the nucleophile-reactive dehydroazepine (Chapter 2, Section 7.1 and Chapter 5, Section 3). However, additional photoreactive groups that also are useful include a psoralen ring system and a benzophenone group. A psoralen-based biotinylation agent is presented in this section, while a benzophenone-containing one with a water-soluble PEG spacer is discussed in Chapter 18, Section 3.6.

4.1. Photobiotin

Perhaps the most common photoreactive biotin derivative is *N*-(4-azido-2-nitrophenyl)-aminopropyl-*N′*-(*N*-D-biotinyl-3-aminopropyl)-*N′*-methyl-1,3-propanediamine, simply called photoactivatable biotin or photobiotin (Forster ***et al.***, 1985) (Thermo Fisher). The compound contains a 9-atom diamine spacer group on the biotin valeric acid side chain at one end, while the other end of the spacer terminates in an aryl azide reactive group. The presence of a nitro group on the phenyl azide ring allows for photoactivation at higher UV wavelengths approaching the visible region of the spectrum, thus avoiding potential breakdown of biological molecules through UV exposure. Photolyzing with light at a wavelength of 350 nm causes rapid activation with nitrene formation. The nitrene can couple to replaceable hydrogen sites in target molecules, add to double bonds within Van der Waals distance, or undergo ring expansion to the dehydroazepine. If ring expansion occurs, the principal target group for coupling is a nucleophile, such as a primary amine (Figure 11.14).



Photobiotin
N-(4-azido-2-nitrophenyl)-aminopropyl-
N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine
MW 533.65
30.0 Å

Photobiotin has been used to biotinylate numerous macromolecules, including proteins and nucleic acids. The biotinylation of alkaline phosphatase was done with complete retention of activity (Forster *et al.*, 1985). Tubulin was labeled with photobiotin and detected on dot blots down to a level of 10 pg of sample using an avidin–enzyme conjugate (Lacey and Grant, 1987). DNA and RNA were labeled for use in hybridization assays (Forster *et al.*, 1985; Keller *et al.*, 1989). For instance, photobiotin-modified probes have been used to detect flavivirum RNA in infected cells (Khan and Wright, 1987), to detect single-copy genes and low-abundance mRNA (McInnes*et al.*, 1987), for the diagnosis of barley yellow dwarf virus (Habili *et al.*, 1987), to assay luteinizing hormone β mRNA in individual gonadotropes (Childs *et al.*, 1987), and to perform DNA mapping using a cross-hybridization technique (Chetrit *et al.*, 1989).

Photobiotin can be dissolved in water or buffer at a concentration of 1 mg/ml and stored in the dark at −20°C until needed. As long as no exposure to light is permitted, the compound is stable for at least 1 year under these conditions.

The protocol for modifying DNA probes with photobiotin can be found in Chapter 27, Section 2.3. It is based on the method of Forster *et al.* (1985). The following method is a suggested protocol for the modification of proteins using a photoreactive biotin derivative. Some optimization may be necessary to obtain the best incorporation levels.

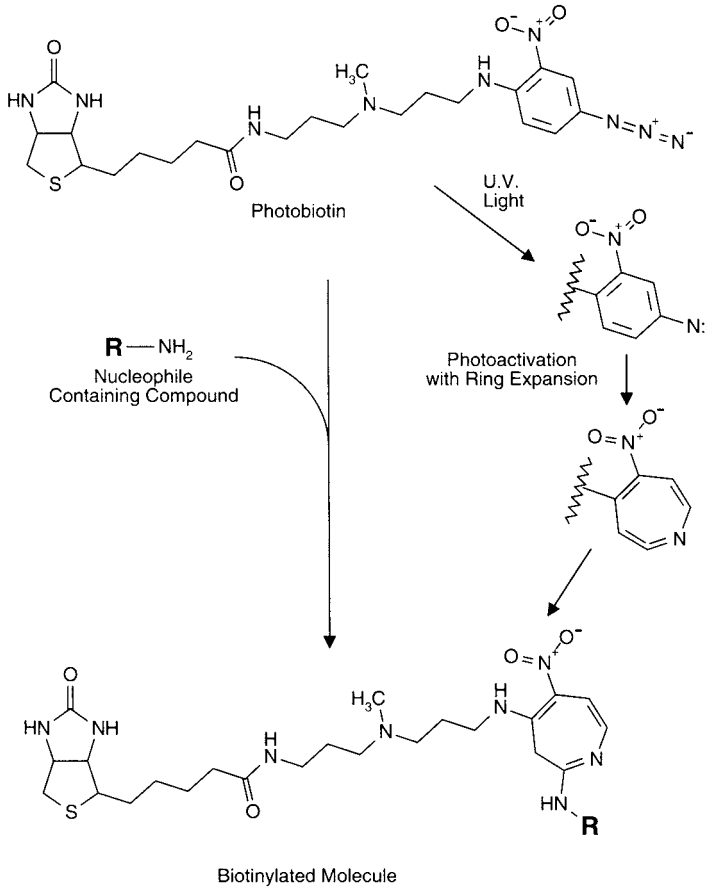


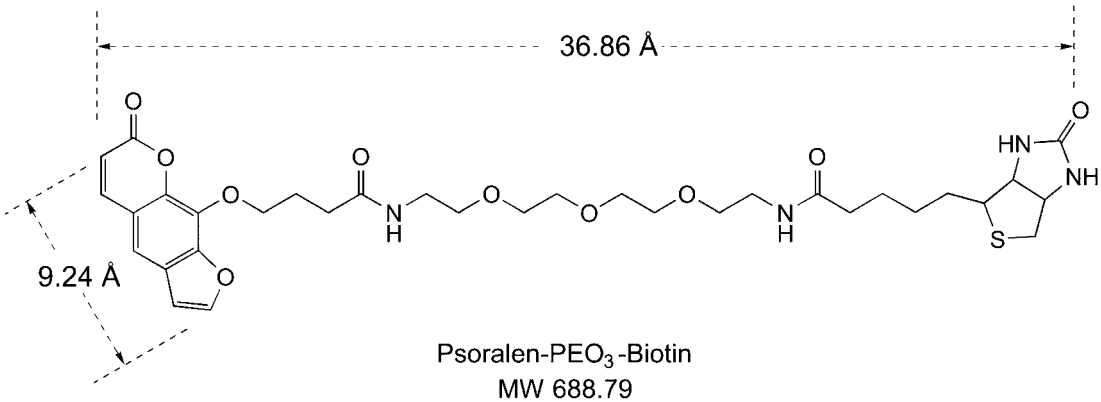
Figure 11.14 Photobiotin can be made to couple spontaneously with nucleophiles by exposure to UV light. The phenyl azide ring undergoes ring expansion to a highly reactive dehydroazepine intermediate, which can react with amines.

Protocol for Labeling Proteins with Photobiotin

1. Dissolve the protein to be biotinylated at a concentration of at least 1 mg/ml in water or dilute buffer at neutral pH.
2. In subdued light, dissolve photobiotin (Thermo Fisher) in water at a concentration of 1 mg/ml.
3. Add a quantity of photobiotin solution to the protein solution to give at least a 5-fold molar excess of biotinylation reagent.
4. Place in an ice bath and irradiate from above (about 10 cm away) for 15 minutes using a sunlamp (such as Philips Ultrapnil MLU 300 W, General Electric sunlamp RSM 275 W, or National Self-Ballasted BHRF 240–250 V 250 W W-P lamp).
5. Remove excess photobiotin by dialysis or gel filtration using a desalting column.

4.2. Psoralen-PEO_3-Biotin

Psoralen-PEO_3-biotin is a photoreactive biotinylation reagent containing a psoralen group at one end and a triethylene glycol (PEG-based) spacer in the middle (Thermo Fisher). This compound is water-soluble due to the presence of the hydrophilic PEG arm. It is able to photoinsert into double-stranded DNA and to a lesser extent into double-stranded regions of RNA. The reaction occurs upon exposure to UV light in the range of 320–400 nm, which forms an excited triplet state intermediate that can insert in certain double bond structures, especially at the 5,6-double bond of thymine bases.



The psoralen ring system can intercalate within double-stranded DNA or RNA and induce the formation of adducts with adjacent thymine bases (Figure 11.15). The furan-side and pyrone-side of the tricyclic rings in psoralen both can form cycloaddition products with the 5,6-double bond of thymine residues, which results in crosslinks between the DNA strands with a PEG-biotin label sticking out.

Psoralen-PEO_3-Biotin has been used to label double-stranded DNA for detection using (strept)avidin reagents (Henriksen *et al.*, 1991; Wygrecka *et al.*, 2007). The psoralen photoreactive group provides better insertion yields than typical phenyl azide-based systems, such as the standard photobiotin probe discussed previously in this section.

Protocol

1. Dissolve the DNA sample to be modified at a concentration of 20–100 μg/ml in 10 mM Tris, 1 mM EDTA, pH 7.4. *Note*: The sample may be heated to denature and solubilize genomic DNA and then cooled to form dsDNA for modification.
2. Dissolve the Psoralen-PEO_3-biotin reagent in DMF at a concentration of 20 mM (use a fume hood). Protect from light.
3. Add a quantity of the Psoralen-PEO_3-biotin solution to the DNA solution to result in a final concentration of 200 μM. Mix well.
4. Expose the solution to long wavelength UV light at about 365 nm (Philips TL 20W/09 UV light works well) for 10–30 minutes. The solution may be cooled on ice to prevent heating during the irradiation process.

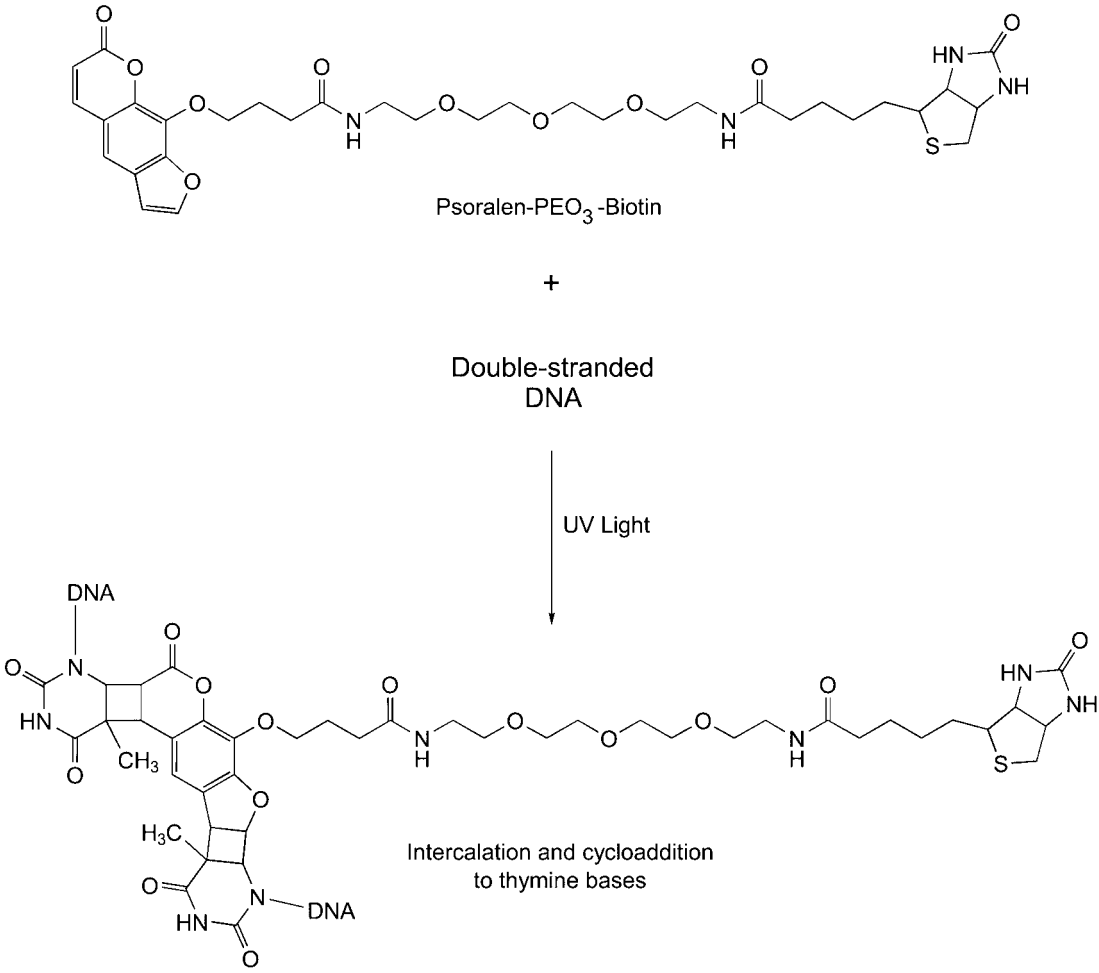


Figure 11.15 The photoreactive compound psoralen-PEO_3-biotin can intercalate into double stranded DNA or RNA segments and covalently link to thymine bases via a photoreaction process.

5. Precipitate the sample to remove unreacted biotinylation reagent by adding 0.1 M potassium acetate and ethanol (1:2 ratio). Centrifuge and wash the biotinylated DNA pellet with ethanol, then dry it under nitrogen. The purified sample may be dissolved in water or buffer.

5. Active Hydrogen-Reactive: *p*-Aminobenzoyl Biocytin, Diazotized

p-Aminobenzoyl biocytin contains a 4-aminobenzoic acid amide derivative off the α-amino group of biocytin's (Section 3, this chapter) lysine residue (Thermo Fisher). The aromatic amine can be treated with sodium nitrite in dilute HCl to form a highly reactive diazonium group (Figure 11.16), which is able to couple with active hydrogen-containing compounds. A diazonium reacts rapidly with histidine or tyrosine residues within proteins, forming covalent diazo

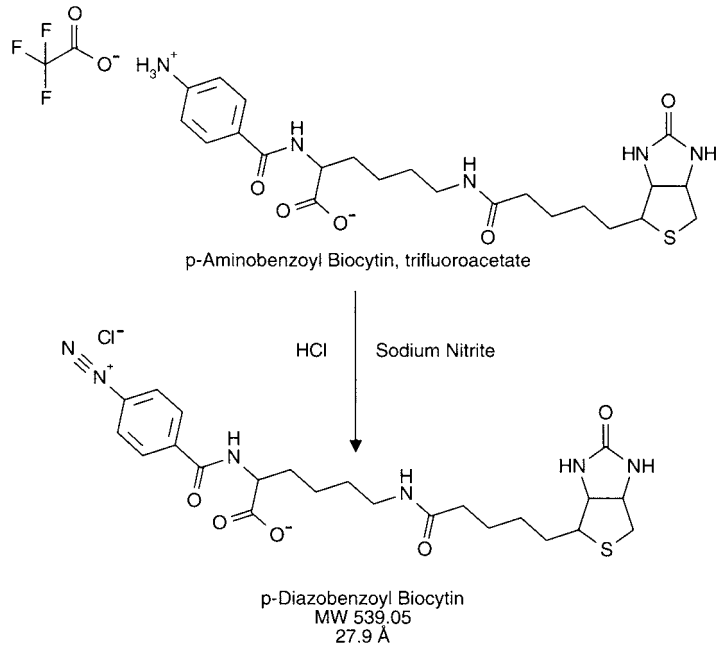
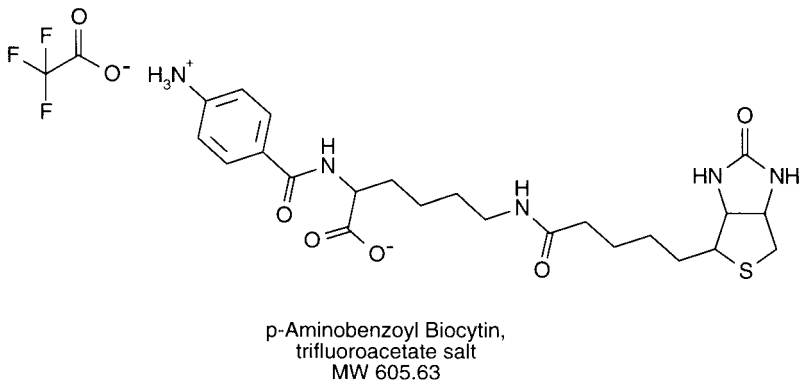


Figure 11.16 The aminophenyl group of this biotin derivative can be transformed into a diazonium reactive group by treatment with sodium nitrite in dilute HCl.

bonds (Wilchek *et al.*, 1986) (Figure 11.17). It also can react with guanidine residues within DNA at position 8 of the base (Rothenberg and Wilchek, 1988) (Figure 11.18). Biotinylation via diazo linkages is reversible by treatment with a 10-fold molar excess of $Na_2S_2O_4$ (sodium dithionite) in 50 mM Tris, pH 8.5 (Gorecki *et al.*, 1971) (Chapter 2, Section 6.1 and Chapter 4, Section 9).



The procedure for creating the diazonium derivative of *p*-aminobenzoyl biocytin and coupling to a protein or a nucleic acid is as follows.

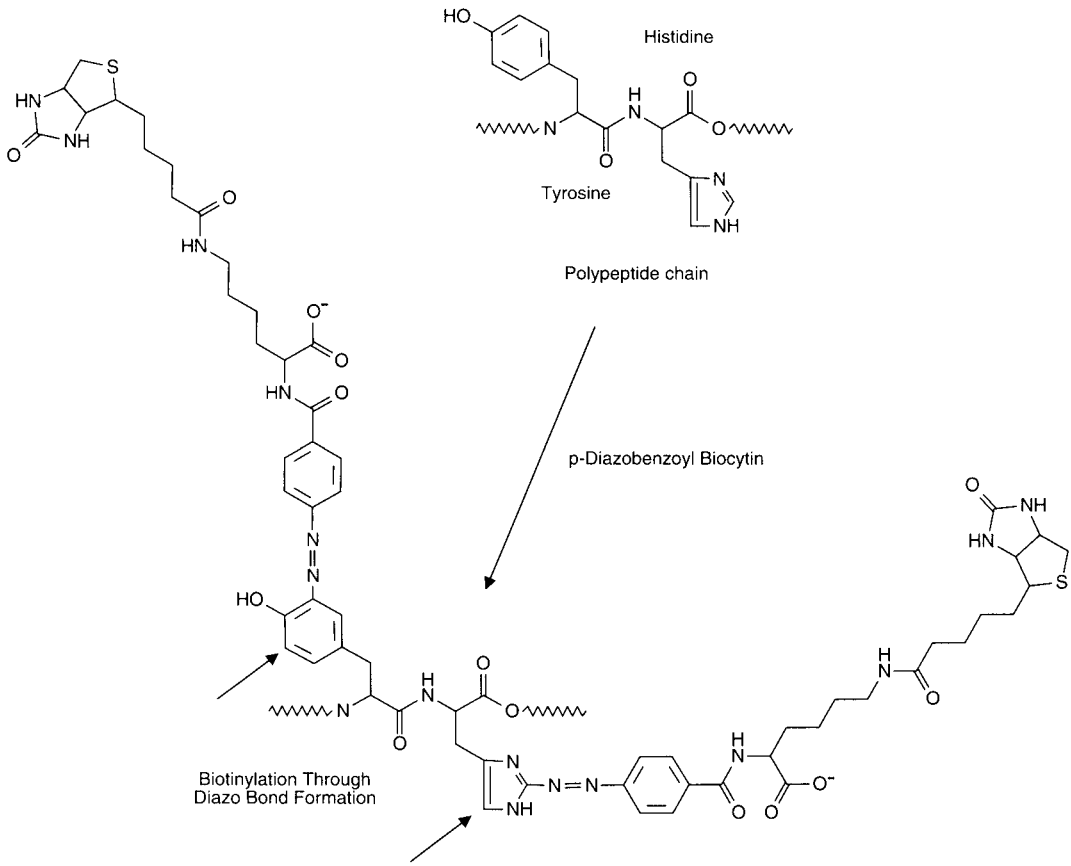


Figure 11.17 The diazonium group of *p*-diazobenzoylbiocytin can react with tyrosine or histidine residues in proteins to form diazo bonds.

Protocol

Formation of the diazonium derivative:

1. Dissolve 2 mg of *p*-aminobenzoyl biocytin (Thermo Fisher) in 40 µl of 1 N HCl (concentration of 50 mg/ml). Cool the solution on ice.
2. Dissolve 7.7 mg of sodium nitrite in 1 ml of ice-cold water. Prepare fresh.
3. Mix 40 µl of the *p*-aminobenzoyl biocytin solution with 40 µl of the sodium nitrite solution.
4. React for 5 minutes on ice to create the diazonium derivative.
5. Stop the reaction by the addition of 35 µl of 1 N NaOH. Use immediately for biotinylation.

Biotinylation of proteins on blots using the diazonium derivative of *p*-aminobenzoyl biocytin.

1. Dilute the diazonium derivative of *p*-aminobenzoyl biocytin with 0.2 M sodium borate, pH 8.4, to a concentration of 10 µg/ml.

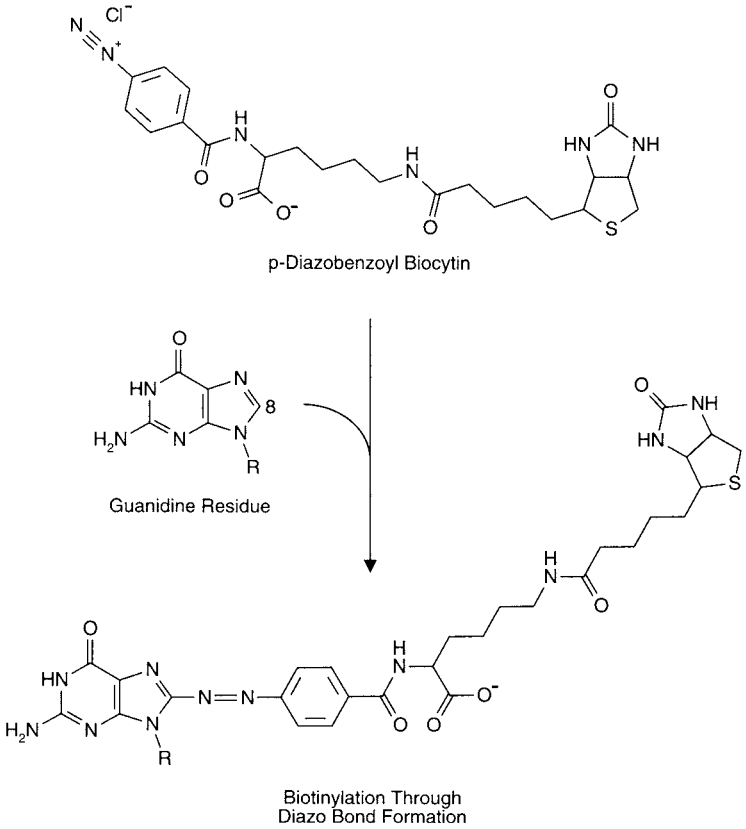


Figure 11.18 The diazonium group of *p*-diazobenzoylbiocytin can couple to the C-8 position of guanidine bases in nucleic acids, forming diazo bonds.

2. Transfer proteins onto a nitrocellulose membrane using any appropriate procedure, including dot blotting the protein solution onto the surface.
3. Incubate the membrane with the biotin derivative at a ratio of 1 ml/cc^3 of membrane.
4. React for 1 hour at room temperature.
5. Wash the membrane thoroughly with 0.1 M Tris, 0.15 M NaCl, pH 7.5.
6. Block nonspecific sites on the membrane with an appropriate blocking component (such as BSA) and detect the biotinylated proteins using an avidin or streptavidin conjugate.

6. Glycan Biotinylation Reagents

Biotinylated oligosaccharides are convenient probes of carbohydrate interactions, because the biotin label can be captured or detected using an avidin or streptavidin derivative. For instance, immobilized streptavidin can be used to purify glycoconjugates that have been labeled

with a biotin group, potentially isolating glycoproteins or carbohydrate binding proteins. Enzyme- or fluorescently-labeled avidin or streptavidin can be used to probe for biotin-labeled carbohydrates in cells or tissue samples. In addition, a biotinylated glycan can be displayed on avidin or streptavidin to make an immunogen for developing specific antibodies to the carbohydrate.

Complex glycans on glycoproteins or other carbohydrate bearing molecules can be modified with a biotinylation reagent using a number of reaction strategies. Oxidation with sodium meta periodate can be used to create aldehyde residues from diols on sugars, and this technique has been used to specifically modify sialic acids on glycans by reductive amination with a biocytin-hydrazide compound (see Section 3.2, this chapter, Bayer *et al.*, 1988). Other procedures make use of released glycans from glycoproteins or other glycoconjugates, which contain reducing ends upon cleavage. The reducing ends then can be reacted with amine- or hydrazide-containing biotinylation compounds to couple with the open aldehyde group at the reducing end, thus forming a hydrazone linkage. The hydrazone bond may be reduced to stabilize the bond (recommended when reacting with amine-containing biotin compounds) or left as the unreduced hydrazone, which typically is done when coupling with hydrazide-biotin compounds. Alternatively, the reducing end of a carbohydrate can be reacted with an amine to form a glycosylamine derivative without opening the acetal ring, thus better preserving the native structure of a glycan, which is important in some studies involving protein interactions.

A recent addition to the methods of glycan biotinylation makes use of the Staudinger ligation reaction with a phosphine-biotin derivative (see also Chapter 17, Section 5). Carbohydrates containing azide derivatives have been modified with this biotin compound to probe for glycoconjugates *in vivo*. This reaction is particularly useful for doing cell-based assays, because the ligation reaction is completely orthogonal to any biological reactions or interactions.

The following sections describe fluorescent biotinylation reagents that can be used to study carbohydrate function and interactions.

6.1. Biotinylated Aminopyridine

BAP (biotinylated aminopyridine or 2-amino-(6-amidobiotinyl)pyridine) is a derivative of D-biotin made by reacting the NHS ester of this vitamin with 2,6-diaminopyridine (DAP) in large molar excess, typically done using a carbodiimide EDC/NHS reaction (Figure 11.19). The resultant compound has fluorescent properties due to the present of the aminopyridine ring, and its remaining free amine group may be used to modify reducing saccharides and glycans by reductive amination (Figure 11.20). BAP can be used to label oligosaccharides under mild conditions and without the carbohydrate structural degradation that results using periodate oxidation of carbohydrates.

Rothenberg *et al.* (1993) demonstrated the utility of BAP for highly sensitive fluorescence detection and separation of oligosaccharides by reverse phase HPLC, with limits of detection down to about the 50-femtomole level (low picomole levels if using a cuvette reader with a 1 cm path length). Toomre and Varki (1994) subsequently published an improvement on the synthesis and use of the BAP reagent. In addition, the biotin group of BAP-labeled glycans can be used to create neoglycoproteins by interaction with tetrameric avidin or streptavidin molecules. The resultant glyco-complexes have been shown to be potent immunogens for evoking an IgG immune response in mice toward the glycan components (Srikrishna *et al.*, 2001).

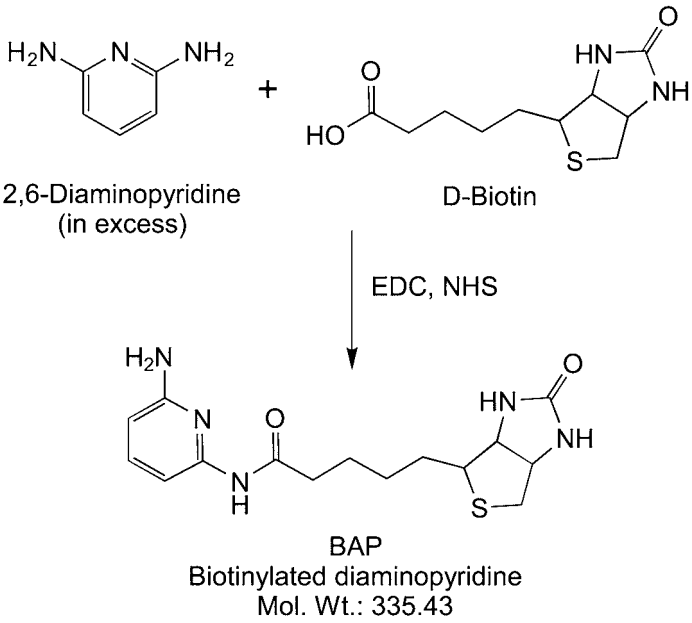


Figure 11.19 The synthesis of BAP can be done by reacting an excess of diaminopyridine with biotin in the presence of EDC and NHS.

BAP-modified glycans also can be used to probe for receptors or binding proteins, which then can be detected by use of streptavidin conjugates or isolated by affinity chromatography on immobilized streptavidin or immobilized monomeric avidin (Thermo Fisher). The use of immobilized monomeric avidin is convenient, because the biotinylated glycans can be released by elution with acid pH or by using a solution containing biotin. BAP-carbohydrate adducts have been shown to be high affinity binders of both streptavidin and avidin, despite the relatively short spacer afforded by the diaminopyridine-biotin linker (Toomre and Varki, 1994).

The biotin group of BAP makes the compound somewhat hydrophobic, but attached to glycans the conjugates should display good water solubility due to the abundance of hydroxyl groups in addition to potentially having other charged groups on the sugars. BAP solubility in water was reported to be about 1 mg/ml, but with the addition of less than 1 percent DMSO, this solubility can be increased more than 10-fold (Toomre and Varki, 1994).

Fluorescence of the diaminopyridine group allows detection of conjugates down to the picomole range, with excitation and emission maxima at 345 and 400 nm, respectively. For detection of BAP and its conjugates, the optimal buffer environment is less than pH 5, because its fluorescent properties are pH dependent. A preferred buffer is sodium acetate at pH 4.

After BAP conjugation to saccharides or glycans, separation of the conjugates and unreacted BAP can be fluorescently followed using size exclusion chromatography (SEC) on a TSK-G3000PW column, which successfully resolves most of the lower molecular weight conjugate species, including single-sugar adducts through 3-sugar carbohydrates. BAP-glycan conjugates containing more than 3 sugars elute early in the separation and do not resolve into discrete peaks, as smaller adducts do. Unreacted BAP elutes last in the SEC separation.

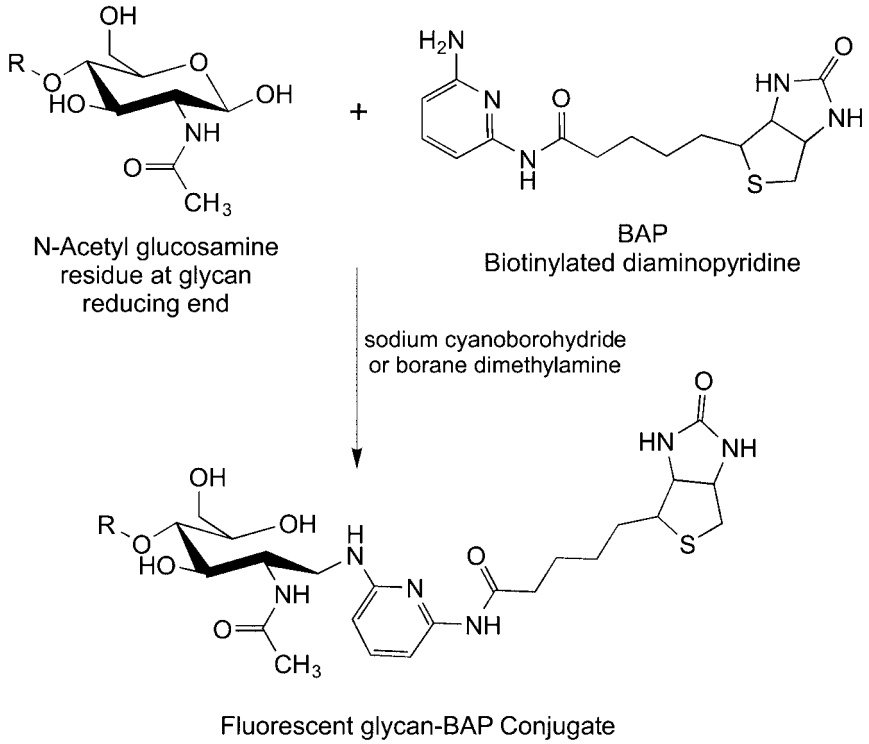


Figure 11.20 BAP can be used to label the reducing end of released glycans by reductive amination in the presence of a reducing agent.

Alternatively, separations can be done by anion-exchange chromatography using a column packed with the HPLC support TSK-DEAE-2SW, which effectively resolves negatively charged carbohydrates, such as those containing sialic acid residues. BAP–glycan conjugates will elute according to their degree of negative charge character. Sulfate-containing sugars in general will interact more strongly with the matrix and have longer retention times than those containing only carboxylates.

BAP may be prepared by the reaction of NHS-biotin with DAP. The biotinylation compound is commercially available (Thermo Fisher) or it may be formed *in situ* by reaction of D-biotin with EDC and NHS. An optimized protocol for the preparation of the reactive intermediate ester and the final BAP compound can be determined from Rothenberg *et al.* (1993) with modifications by Toomre and Varki (1994). Basically, a solution of 0.3 M DAP is prepared in 40 ml of 50 mM MES, pH 6.5, and 10 ml of 0.1 M D-biotin in DMSO is added. The reaction is initiated by the addition of EDC and NHS to a final concentration of 150 and 50 mM, respectively. The reaction was allowed to continue overnight at room temperature with mixing before purification of BAP on a C_{18} sample prep cartridge. The reaction mixture was applied to the cartridge and reaction by-products and DAP were removed by washing with water and 10 percent acetonitrile. BAP was finally eluted in high purity by washing with 50 percent acetonitrile.

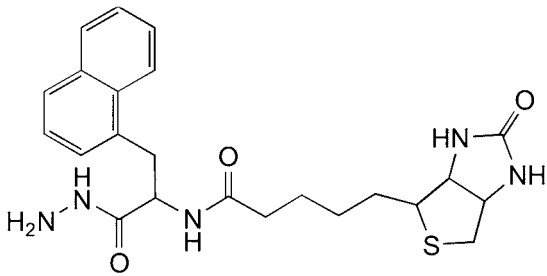
The conjugation of BAP to oligosaccharides can be done by the following protocol based on the method of Toomre and Varki (1994).

Protocol

1. Dissolve an oligosaccharide or glycan having a reducing end to be modified in 2:1 pyridine/glacial acetic acid (vol/vol) with a total reaction volume of 10–100 μl. If the carbohydrate initially is insoluble in the reaction solution, a prior dissolution in a minimal amount of DMSO or water can be done and then an aliquot transferred to the reaction medium.
2. Add to the solution a 50-fold molar excess of BAP over the estimated amount of carbohydrate present in the reaction mixture.
3. Heat at 80°C in a sealed Reactivial (Thermo Fisher) for 1 hour.
4. Add to the reaction mixture an equal volume of the reducing agent borane-dimethylamine (BDA) complex, which was previously prepared in the reaction buffer at a concentration of 125 mg/ml.
5. React for another hour at 80°C.
6. Purify the BAP–glycan conjugate from unreacted glycans by use of a C_{18} sample prep cartridge, as described above for the synthesis of BAP. Separation of excess BAP from the conjugates may be done by SEC or anion exchange chromatography, depending on the size of carbohydrates being modified and their intrinsic charge. Follow the separations by visualization of BAP fluorescence with a hand-held UV lamp or through the use of a fluorescence detector.

6.2. Biotinyl-L-3-(2-naphthyl)-alanine hydrazide

BNAH is a biotin-hydrazide derivative containing a UV absorbing and fluorescent naphthalene group (biotinyl-L-3-(2-naphthyl)-alanine hydrazide) (Leteux *et al.*, 1998). Unlike BAP described previously, BNAH has a hydrazide group for coupling to the reducing end of carbohydrates, instead of an amine. While both groups can be successfully conjugated to an aldehyde of a released glycan, the biotin–amine compounds require a reducing agent to stabilize the resultant hydrazone bond.



BNAH
Biotinyl-L-3-(2-naphthyl)-alanine hydrazide
Mol. Wt.: 455.57

BNAH may be coupled to reducing sugars without reduction, since the linkage formed between a hydrazide and an aldehyde is much more stable than that with an amine.

In some cases, the ability to modify glycans at the reducing end without reduction preserves the carbohydrate's native structure sufficiently to allow interactions with proteins that would otherwise not interact if the bond were reduced. Therefore, depending on the ultimate use of the biotinylated carbohydrate, using a hydrazide mediated conjugation process can have advantages over the use of amine-biotin compounds.

In the case of BNAH, however, it was determined that the resultant linkage with the reducing end of an oligosaccharide or glycan was not a hydrazone bond, but a glycosylhydrazide derivative, which preserves the pyranose ring structure of the sugar (Figure 11.21). This finding is the main reason a BNAH modified glycan effectively displays a near-native conformation at the reducing end. In addition, the biotin label in this configuration is reversible and can be released by incubation under acidic conditions, thus allowing recovery of the carbohydrate.

Another advantage of the BNAH derivative is that the conjugation reaction with reducing sugars can be done in aqueous conditions and in an environment that permits carbohydrate and biotinylation reagent solubility. Modified carbohydrates may be stored for at least 1 year at −20°C in a solution of water/methanol (9:1 v/v) without degradation.

The following protocol is based on the method of Leteux *et al.* (1998).

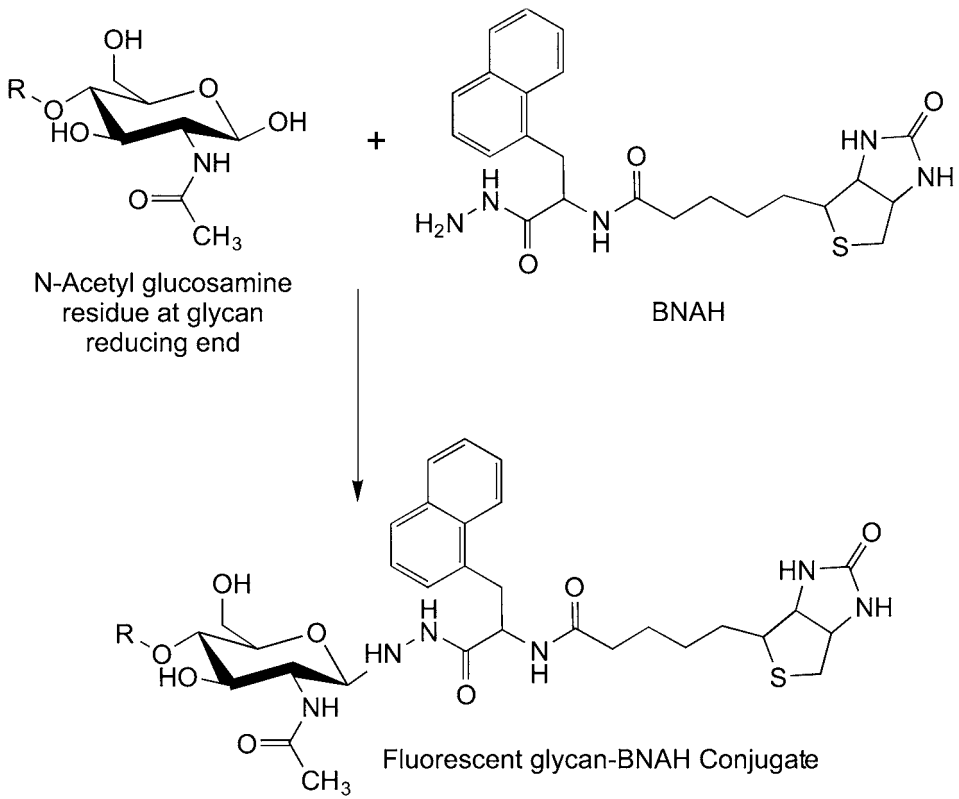


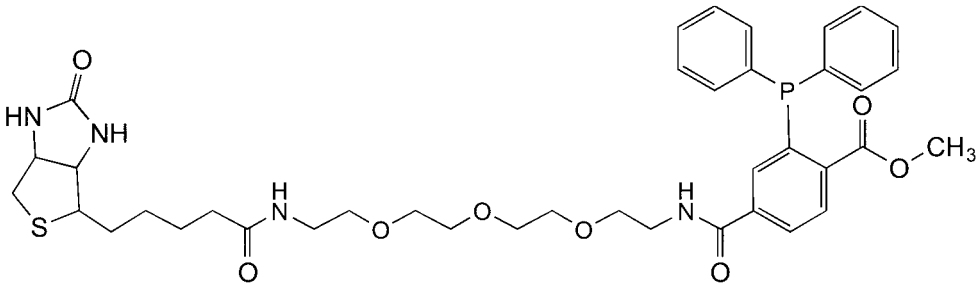
Figure 11.21 BNAH contains a hydrazide group that can be used to label the reducing end of released glycans through the formation of a hydrazone bond.

Protocol

1. Dissolve 100 nmol of the carbohydrate to be modified and 500 nmol of BNAH in 25 µl of methanol (20 mM BNAH solution).
2. Evaporate the solution to dryness and re-dissolve in 25 µl of either an acidic solution of methanol/water/acetic acid (74:8:8, v/v) or in a neutral solution consisting of methanol/water (9:1, v/v), depending on the relative solubility of the carbohydrate.
3. React for at least 5 hours at 60°C if using the acidic reaction solution or for a total of 16 hours at 60°C if using the neutral solution.
4. Purification of the conjugates may be done by reverse phase HPLC separation. Dry the reaction solution under a nitrogen stream and reconstitute in a minimum volume of acetonitrile/water (1:1, v/v). Apply the sample to a 5 µm C_{18}-silica HPLC column (250 × 4.6 mm, Nucleosil). Elute with a gradient of water to acetonitrile at a flow rate of 1 ml/minute over a time course of 30 minutes. Free BNAH and BNAH-glycan derivatives can be monitored by absorbance at 275 nm. The conjugate peak also will be positive for carbohydrate by reaction with orcinol, which can be detected by spray after spotting a small eluted sample on a TLC plate.

6.3. Biotin-PEG-Phosphine

Saxon and Bertozzi (2000) reported on the synthesis and use of a novel biotinylation compound containing a phosphine group for coupling to azide-containing molecules. The reagent has a biotin handle at one end, a tetraethyleneglycol (PEG) spacer imparting increased water solubility in the middle, and a 3-(diphenylphosphino)-4-(methoxycarbonyl)benzamide group on the other end. Biotin-PEG-phosphine reacts with azide derivatives of amino acids, sugars, and cross-linkers to form an intermediate aza-ylide, which spontaneously rearranges in aqueous solution to create a stable amide bond (Figure 11.22). This reaction is a modified Staudinger ligation that can be used to target azide containing glycans or proteins *in vivo*.



Biotin-PEG-Phosphine
Mol. Wt.: 764.87

Cells grown in the presence of azide analogs of certain amino acids or sugars will incorporate these derivatives into proteins or carbohydrates through enzymatic synthesis using

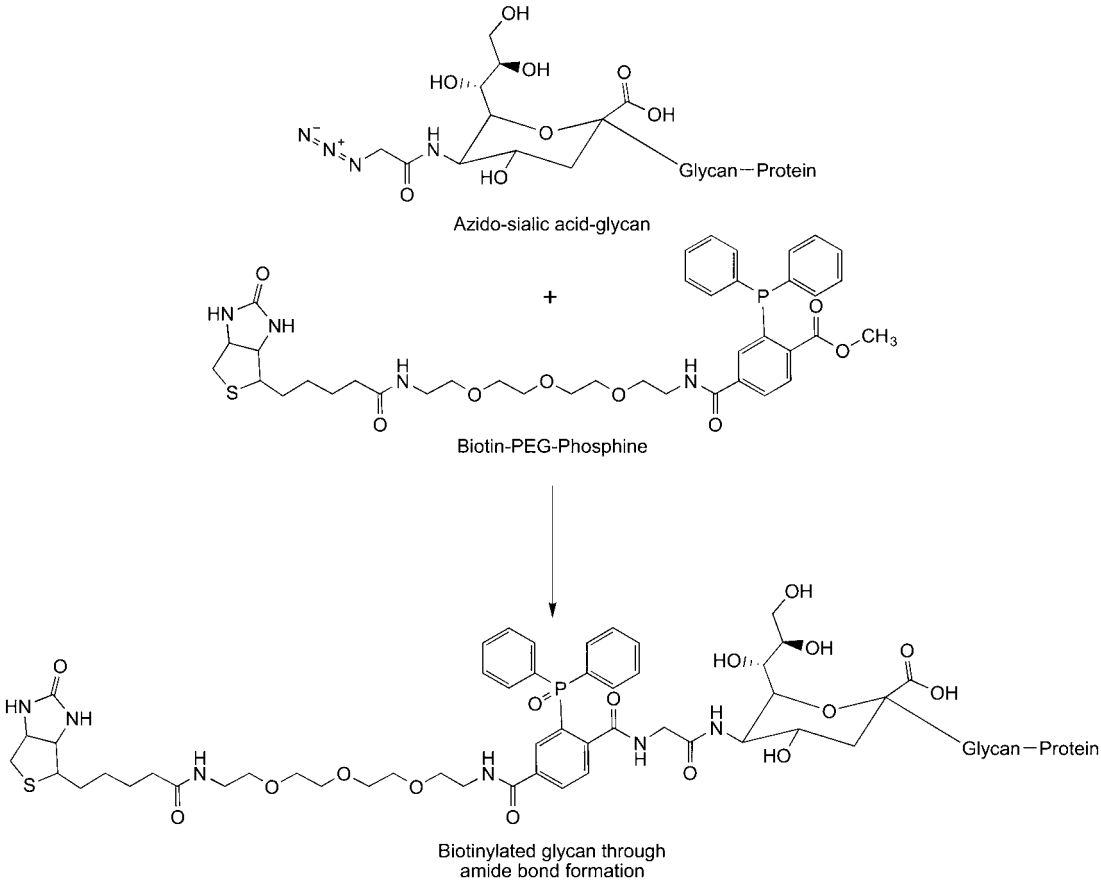


Figure 11.22 Azido-sialic acid-containing glycans can be labeled *in vivo* with biotin-PEG-phosphine using the Staudinger ligation reaction, which forms an amide bond.

the native cell machinery. Azides thus displayed on biomolecules are unreactive with other substances typically found within the cell, but the azide derivatives may be targeted for conjugation using the modified Staudinger reaction (see Chapter 17, Section 5 for additional information on this reaction and its use in biological labeling).

Biotin-PEG phosphine can be used to label glycans or proteins that have been modified to contain azide groups. The beautiful specificity of this reaction and its lack of toxic side reactions or additives make it suitable for use in living organisms, including cells and animals. The reaction is completely orthogonal to functional groups and reactions found in living systems, so the biotinylation process proceeds with no cross-reactions with other biomolecules. In addition, no cell toxicity has been observed due to the phosphine or the phosphine oxide by-product of the coupling reaction. The phosphine also doesn't appear to be capable of reducing disulfides within proteins.

Prescher *et al.* (2004) have shown that mice fed with the peracetylated azido-mannose sugar derivative Ac_4ManNAz efficiently convert it through deacetylation by cytosolic esterases into

an azido sialic acid (SiaNAz), which then gets incorporated enzymatically into cell-surface glycans. Biotinylation of these aberrant carbohydrates provides a method of detecting or isolating glycoproteins or other glycoconjugates. For instance, fluorescently labeled streptavidin can be used to image the cell-surface structures containing the biotinylated azido-sugar derivatives. Alternatively, immobilized streptavidin or immobilized monomeric avidin can be used to purify biotinylated azido-glycoconjugates after cell lysis and provide a method for studying glycoprotein function and interactions.

The following protocol is based on the methods of Saxon and Bertozzi (2000) and Prescher *et al.* (2004).

Protocol

1. Treat and grow cells in the presence of 20 μM Ac_4ManNAz for at least 3 days. The azide-mannose derivative may be solubilized in 70 percent DMSO as a more concentrated stock solution and then an aliquot added to the media containing the cells. Alternatively, mice may be treated with the azide-sugar derivative in aqueous DMSO at a level of 100–300 mg/kg, using an injection of 200 μl administered intraperitoneally daily for 7 days.
2. When working with cells, first wash them several times with PBS, pH 7.4, to remove any remaining Ac_4ManNAz from the media. Alternatively, if working with animals, isolate the tissue type desired and prepare the cells to be labeled in PBS, pH 7.4.
3. To label cell-surface azide-glycans, the cells are reacted for 1 hour using a final concentration of 1 mM biotin-PEG-phosphine reagent dissolved in PBS, pH 7.4.
4. Wash the cells several times with PBS, pH 7.4, to remove excess biotinylation compound.
5. The labeled glycans may be analyzed by cell sorting after staining with fluorescently modified streptavidin. Alternatively, the cells may be lysed and the labeled glycans isolated using immobilized streptavidin.

12

Iodination Reagents

Modification of proteins and other molecules with a radioactive element provides a means of detection that can be extremely sensitive for assay, localization, and imaging applications. Among the most common radiolabels for biological studies are ^{14}C, ^{32}P, ^{35}S, ^{3}H, and the isotopes of iodine, ^{125}I and ^{131}I. The unstable isotopes of carbon, phosphorus, sulfur, and hydrogen are all β emitters, releasing particulate radiation consisting of either positrons or electrons. To measure labeled molecules containing β emitters often necessitates tedious sample manipulation including tissue homogenization and mixing with scintillation cocktails for subsequent counting.

The radioactive isotopes of iodine, by contrast, are both γ emitters, providing a much easier route to measurement than β-particle-emitting radioisotopes. High-energy electromagnetic radiation can be detected directly without the need for intermediate scintillation cocktails. Iodine-131 was the first unstable iodine isotope to be used for labeling protein molecules (Li, 1945; Pressman and Keighley, 1948). The ^{131}I isotope decays by both β^- (electron) and γ emission. The specific activity of this element can be as high as 6,550 Ci/mmol, providing extraordinary sensitivity for detecting labeled molecules.

Iodine-125 decays by electron capture followed by γ emission. However, the maximum energy of ^{125}I electromagnetic energy emission can be as little as one-tenth to one-third that of ^{131}I (Wilbur, 1992; Powsner, 1994). The greater energy intensity of ^{131}I emission actually can be a disadvantage, since γ rays emanating from it are more penetrating, requiring increased precautions and greater protective equipment. In addition, the relatively short half-life of ^{131}I (8.1 days) as compared to ^{125}I (60 days) necessitates that labeled compounds be prepared more often, since activity losses will be severe upon storage. Because ^{125}I is not a particle emitter, its use *in vivo* for imaging applications limits radiation damage to surrounding proteins, cells, and tissues.

These factors make ^{125}I the iodine label of choice for radiolabeling biological molecules. Its commercial availability from a number of suppliers at relatively low cost further adds to its popularity. Even though it has lower specific activity than ^{131}I, iodine-125 still provides much greater sensitivity than ^{14}C, ^{32}P, ^{35}S, or ^{3}H in labeling biomolecules. In fact, the use of a radioactive iodine label can create probes that have 150-fold more sensitivity than tritiated molecules and as much as 35,000 times the detectability of ^{14}C-labeled molecules (Bolton and Hunter, 1986).

Radioiodination is the process of chemically modifying a molecule to contain one or more atoms of radioactive iodine. Early studies on protein modification determined that iodine in

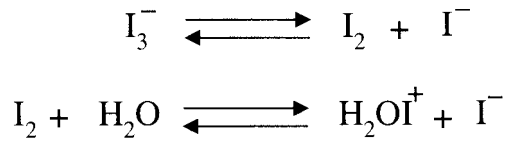


Figure 12.1 Iodide anion in aqueous solution undergoes an equilibrium reaction process to form the reactive H_2OI^+ species.

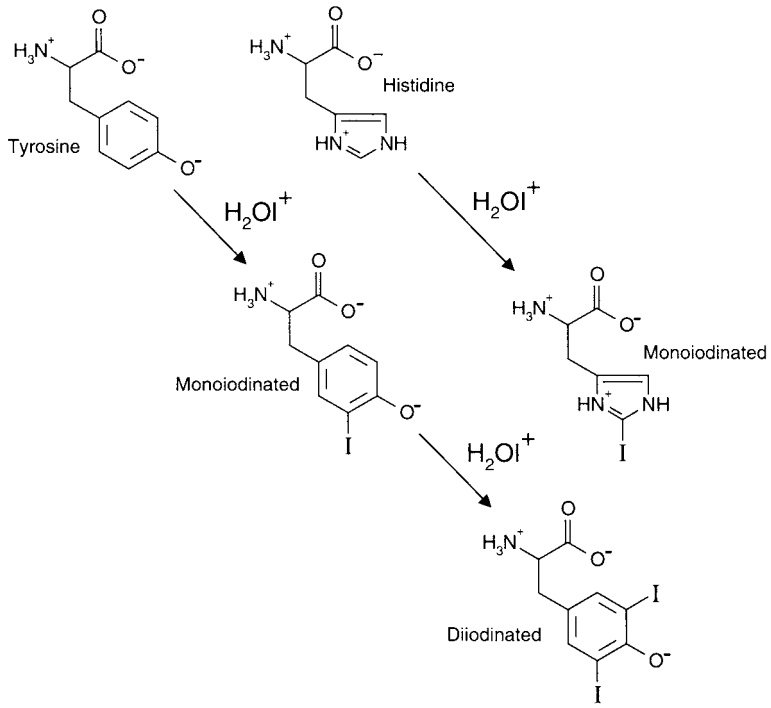


Figure 12.2 The iodination of tyrosine or histidine residues in proteins by H_2OI^+.

aqueous solution formed a reactive ion, H_2OI^+ (Figure 12.1), that is capable of modifying tyrosine side chains, the imidazole groups of histidine, and either modifying sulfhydryl groups or catalyzing their oxidation to disulfides (Figure 12.2). Most methods now utilize a chemical agent to create the reactive iodine species, thus driving the reaction at much greater rates.

There are two main methods of radioiodination that are commonly employed to modify proteins and other molecules: (1) direct labeling of the desired protein or other target molecule in the presence of an oxidizing agent or (2) indirect labeling of the desired molecule by first labeling an intermediate compound which is then used to perform the final modification. Direct labeling methods are by far the most common, and the chemistries used in this process have been reviewed (Regoeczi, 1984).

The prevailing procedures for direct coupling of ^{125}I to a protein or other molecule are through the use of oxidizing agents. The *in situ* preparation of an electrophilic radioiodine

species is fundamental to the ability to modify certain reactive sites within the desired molecules. The most common oxidizing compounds are *N*-haloamine derivatives, such as *N*-chlorotoluenesulfonamide (chloramine-T) or 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen). In most instances, such compounds do not harm the proteins being labeled, although careful control over reaction times should be done to prevent over-labeling or oxidative damage. A secondary method of producing an oxidative effect is to use an enzyme-driven system. The glucose oxidase/lactoperoxidase reaction creates reactive iodine through the production of hydrogen peroxide from glucose with the subsequent action of peroxidase to form I_2 from I^-.

Formation of the electrophilic halogen species leads to the potential for rapid reaction with compounds containing strongly activating groups, such as in activated aryl compounds. Particularly, substances containing aromatic ring structures that have substituents on the ring which are electron donating can sufficiently activate the carbons on the ring to undergo electrophilic substitution reactions. Therefore, phenols, aniline derivatives, or alkyl anilines that contain OH, NH_2, or NHR constituents respectively, are very susceptible to being iodinated. In proteins, this translates into tyrosine side chain phenolic groups and histidine side chain imidazole groups. Crosslinking compounds or modification reagents containing ring-activated groups also are capable of being iodinated.

The addition of a radioactive iodine atom to a protein molecule typically has little effect on the resultant protein activity, unless the active center is modified in the process. The size of an iodine atom is relatively small and does not result in many steric problems with large molecules. The sites of potential protein modification are tyrosine and histidine side chains. Tyrosine may be modified with a total of two iodine atoms per phenolate group, whereas histidine can incorporate one iodine. Sulfhydryl modification at cysteine residues is typically unstable.

The result of iodination at tyrosine groups can alter the spectral characteristics of the protein in solution (Hughes, 1950). The typical protein absorbency at 280 nm can shift to a maximum at about 305–315 nm due to the addition of iodine atoms to the phenolate ring of tyrosine. The degree of absorbance shift is dependent on how many iodine atoms are incorporated into the protein and whether they result in mainly mono- or di-iodotyrosine formation. In addition, as the level of iodination increases, the solubility of a protein in aqueous solution can dramatically decrease until complete insolubility results in proteins with high numbers of tyrosines.

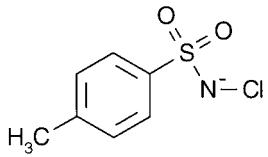
Thus, controlling the degree of iodination is an important consideration both in choosing the oxidant used and in controlling the time of reaction. Typically, most radiohalogenations are done in a time period of 30 seconds to as long as 30 minutes. Optimization may have to be done to determine the correct time to use for a particular modification reaction. Termination of the iodination reaction may be done through addition of a reducing agent, such as sodium metabisulfite. Bisulfite reduces the electrophilic iodine species to unreactive iodide, effectively stopping the modification process.

The following sections discuss the major radioiodination reagents available for direct labeling as well as the main crosslinkers or modification reagents used for indirect labeling techniques.

1. Chloramine-T

Chloramine-T, or *N*-chlorotoluenesulfonamide, has been one of the most popular oxidizing reagents used for radioiodination techniques since its introduction by Greenwood *et al*. in 1963 (Sigma). It has strong oxidizing properties that readily lead to the formation of the required

electrophilic halogen species that result in iodine incorporation into target molecules. The reactions of chloramine-T are well documented, being suitable for both macromolecular protein iodination and small-molecule modification (Wilbur, 1992). It also can be used to modify molecules with other radioactive halogen elements, such as isotopes of bromine and astatine (Hadi *et al.*, 1979; Mazaitis *et al.*, 1981).



Chloramine-T
N-chlorotoluenesulfonamide

The reaction of chloramine-T with iodide ion in solution results in oxidation with subsequent formation of a reactive, mixed halogen species, ICl (Figure 12.3). Either ^{125}I or ^{131}I can be used in this reaction. The ICl then rapidly reacts with any sites within target molecules that can undergo electrophilic substitution reactions. Within proteins, any tyrosine and histidine side chain groups can be modified with iodine within 30 seconds to 30 minutes. Since chloramine-T is a water-soluble reagent and the reaction is done completely in the solution phase, higher incorporation of radioactive iodine can be obtained than using insoluble or immobilized oxidants (see subsequent sections). However, a greater yield of specific radioactivity does not

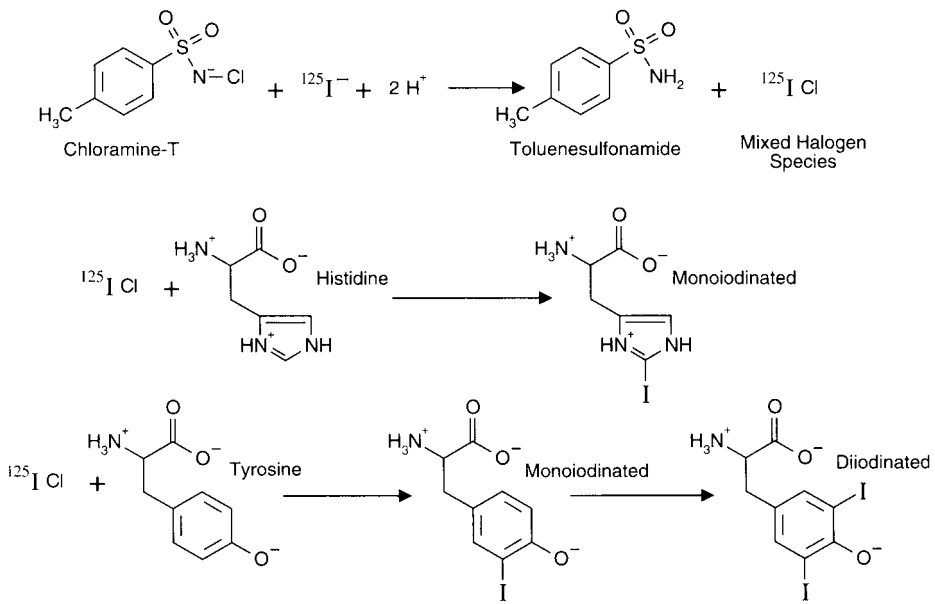


Figure 12.3 The strong oxidant chloramine-T can react with iodide anion in aqueous solution to form a highly reactive mixed halogen species. ^{125}ICl then can modify tyrosine and histidine groups in proteins to form radiolabeled products.

always translate into a better radiolabeled probe. Chloramine-T, being a strong oxidant with rapid reaction rates can easily over-label a target molecule or cause oxidative damage to sensitive proteins (Lee and Griffiths, 1984). The reaction may be quenched with a reductant, usually done by addition of sodium metabisulfite. Although chloramine-T is still widely used, alternative iodination reagents that are insoluble or immobilized on insoluble supports provide milder reaction conditions and are more controllable.

The following protocol is representative of those found in the literature for iodination of protein molecules using chloramine-T.

Protocol

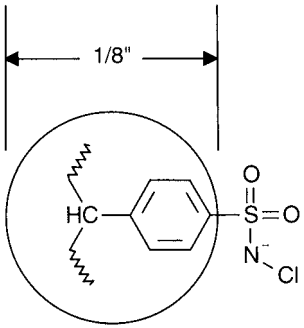
Caution: Handle all radioactive substances according to the radiation safety regulations instituted at each facility approved to handle such materials. Use adequate precautions to protect personal safety and the environment. Dispose of radioactive waste only by following approved guidelines.

1. Dissolve chloramine-T in 50 mM sodium phosphate, pH 7.0, at a concentration of 4 mg/ml. Prepare fresh. Approximately 25 µl of this solution (100 µg) is required to iodinate 5 µg of a protein.
2. Dissolve sodium metabisulfite in 50 mM sodium phosphate, pH 7.0, at a concentration of 12.6 mM (240 µg/100 µl). Prepare fresh. Approximately 100 µl of this solution is required for a 5 µg protein iodination.
3. Obtain fresh $Na^{125}I$ and adjust its concentration to approximately 0.5 mCi/µl; 2 µl of this solution are required to iodinate 5 µg of protein.
4. Add to a suitable reaction vial, 25 µl of a solution consisting of 5 µg of a protein dissolved in 50 mM sodium phosphate, pH 7.0. Mix using a small magnetic stirring chip.
5. Add 2 µl of the $Na^{125}I$ solution (about 1 mCi) to the protein in the vial. Seal the vial using a screw-cap septum that can be penetrated with a syringe.
6. Using a syringe, add 25 µl of the chloramine-T solution and continue to mix for at least 30 seconds. Longer reaction times can be done, but the solution-phase iodination usually proceeds very rapidly.
7. Add 100 µl of the sodium metabisulfite solution to the iodination reaction to stop it. Stir for 10 seconds.
8. Purify the iodinated protein from excess reactants by gel filtration using a desalting resin. The column may be pre-treated by passing a solution of bovine serum albumin (BSA) through it to eliminate nonspecific binding sites that could cause significant protein loss in small-sample applications.

2. Iodobeads

Iodobeads (Thermo Fisher) is an immobilized preparation of a chloramine-T analog, consisting of nonporous, polystyrene beads of diameter 1/8″ that have been derivatized to contain *N*-chlorobenzenesulfonamide groups (as the sodium salt) (refer to US patents 4,448,764 and 4,436,718). During the manufacturing process, the hydrophobic nature of the polystyrene is changed to a rather hydrophilic surface due to the chlorosulfonamide modifications. The surface

character results in excellent protein recoveries (typically greater than 90 percent). The oxidizing capability of Iodobeads is limited to surface reactions on the outer shell of the nonporous polystyrene ball. The effect is to reduce the rate of iodine incorporation into macromolecules from the extremely rapid 30-second reaction of soluble chloramine-T to a more relaxed pace of about 2–15 minutes. This also creates a milder oxidizing environment, thus minimizing the potential for protein degradation or activity loss. A slower iodination process allows more control over the level of iodine derivatization. Often, tyrosine iodination can be limited to mono-iodo forms, avoiding the detrimental effects on solubility or activity that excessive modification can cause.



IODO-BEADS Containing
N-Chlorobenzenesulfonamide
Groups on a Polystyrene Backbone

Markwell (1982) reported that the reaction mechanism for creating the electrophilic iodine species may be somewhat different for Iodobeads than other oxidizing agents. It was demonstrated that the active component remained at or near the surface of the beads during the course of the iodination process. Markwell speculated that an intermediate reactive species, *N*-iodobenzenesulfonamide, is formed from substitution of the chlorine atoms on the bead (Figure 12.4). It is possibly that this intermediate is involved in the direct iodination of target molecules that approach the bead surface.

Iodobeads may be used in a variety of buffer salts and in the presence of detergents or denaturants without affecting iodination. The reagent, however, is susceptible to inactivation by reducing agents such as disulfide reductants, and it can be inactivated by moisture upon storage. Also, avoid organic solvents that can dissolve or affect the surface characteristics of polystyrene, such as dimethylformamide (DMF) or dimethyl sulfoxide (DMSO).

To determine the optimal reaction time for a particular radioiodination, 5 µl aliquots of the reaction medium can be removed every 30 seconds, diluted 1:20,000 with 20 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, containing 0.5 mg/ml BSA as a carrier protein. Finally, precipitate a small amount of the diluted aliquot with trichloroacetic acid (TCA, 60 percent), centrifuge to recover the pellet, wash the pellet once with TCA, and measure the amount of radioactivity in the pellet and supernatant using a gamma counter. The reaction period representing optimal radiolabel incorporation should be used for subsequent radioiodinations.

Directing the iodination reaction toward histidine residues in proteins, as opposed to principally tyrosine modification, is possible simply by increasing the pH of the Iodobeads reaction from the manufacturer's recommended pH 7.0–8.2 (Tsomides *et al.*, 1991). No reducing agent is required to stop the iodination reaction as is the case with chloramine-T and other methods.

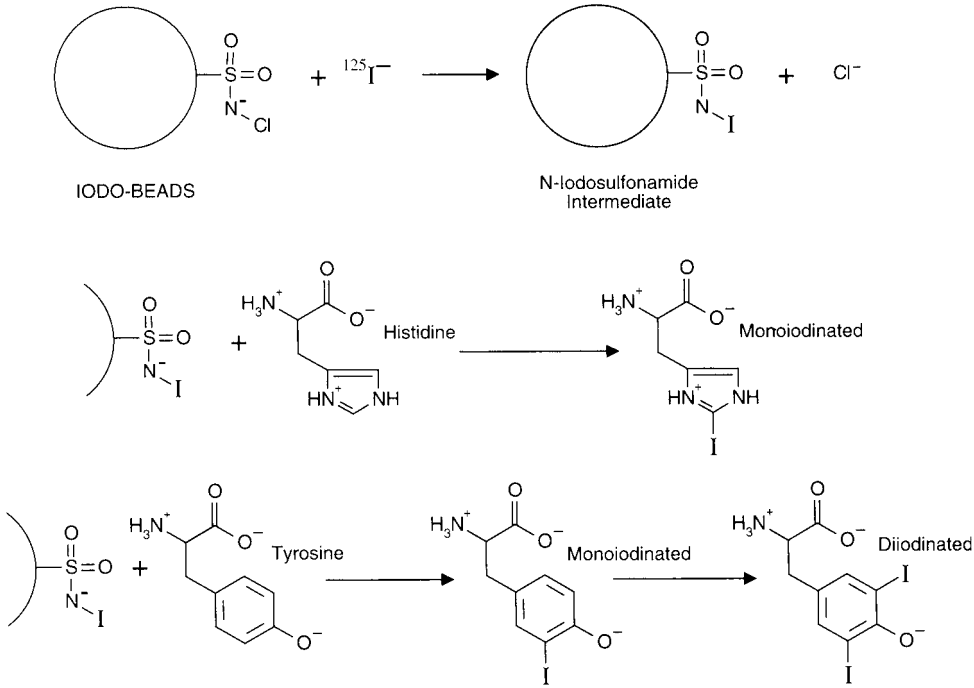


Figure 12.4 IODO-BEADS contains immobilized chloramine-T groups that can react with radioactive iodide in aqueous solution to form a highly reactive intermediate. The active species may be an iodosulfonamide derivative, which then can iodinate tyrosine or histidine residues in proteins.

Simple removal of the bead(s) from the reaction is enough to eliminate the iodination process. The mild nature of the Iodobeads iodination reaction can result in better recovery of active protein than using soluble oxidants (Lee and Griffiths, 1984).

Each bead can iodinate up to 500 μg of tyrosine-containing protein or peptide. This translates into an oxidative capacity of about 0.55 μmol per bead. The rate of reaction can be controlled by changing the number of beads that are used and altering the sodium iodide concentration added to the reaction. Reaction volumes of 100–1,000 μl are possible per bead. The following protocol is suggested for iodinating proteins. Optimization should be done to determine the best incorporation level to obtain good radiolabel incorporation with retention of protein activity.

Protocol

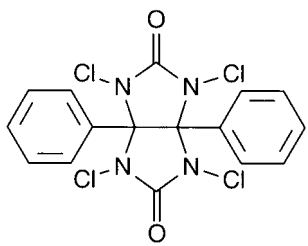
Caution: Handle all radioactive substances according to the radiation safety regulations instituted at each facility approved to handle such materials. Use adequate precautions to protect personal safety and the environment from contamination. Dispose of radioactive waste by following approved guidelines.

1. Wash one or more Iodobeads (Thermo Fisher) with the iodination buffer of choice. Buffers containing 0.1 M sodium phosphate or 0.1 M Tris at slightly acidic to slightly alkaline pH work well. A buffer consisting of 0.1 M sodium phosphate, pH 6.5, will give the highest possible reaction rates and yields.

2. Add the desired number of beads to a solution of carrier-free $Na^{125}I$ in iodination buffer at a concentration level of about 1 mCi per 100 μg of protein to be modified. The total reaction volume should be 100–1,000 μl per bead.
3. Add from 5 μg to 500 μg of a tyrosine-containing peptide or protein dissolved in iodination buffer to the reaction mixture.
4. React for 2–15 minutes at room temperature. Reactions done at 4°C are possible, but will result in slightly lower incorporation of iodine.
5. Stop the reaction by removing the solution from the beads. This can be done by simply pipetting the solution away from the beads or by physically removing the beads. The beads may be washed once with iodination buffer to assure complete recovery of protein. Exact timing of the reaction is important to obtain reproducible results.
6. Remove excess ^{125}I from the iodinated protein by gel filtration using a desalting resin.

3. Iodogen

Iodogen (Thermo Fisher), first described by Fraker and Speck in 1978, is 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril, an *N*-haloamine derivative with oxidizing properties similar to those of Iodobeads and chloramine-T. The compound is insoluble in aqueous solution, therefore making it a type of solid-phase radioiodination reagent. However, unlike Iodobeads wherein the oxidizing group is immobilized on another support material, Iodogen must be plated out on the surface of a reaction vessel prior to doing an iodination. Due to the reagent's stability, the plated reaction vessels can be prepared well in advance and stored in a desiccator until needed (Markwell and Fox, 1978). Alternatively, pre-coated tubes now are available (Thermo Fisher).



IODO–GEN
1,3,4,6-Tetrachloro-
3α,6α-diphenylglycouril
MW 432.09

The reaction of Iodogen with iodide ion in solution results in oxidation with subsequent formation of a reactive, mixed halogen species, ICl (Figure 12.5). Either ^{125}I or ^{131}I can be used in this reaction. The ICl then rapidly reacts with any sites within target molecules that can undergo electrophilic substitution reactions. Within proteins, any tyrosine and histidine side chain groups can be modified with iodine within 30 seconds to 30 minutes. In addition, crosslinking or modification reagents possessing phenyl rings with activating groups present (e.g., electron donating constituents: —OH, —NH_2, etc.) can be iodinated using Iodogen. The incidence of side reactions appears to be negligible.

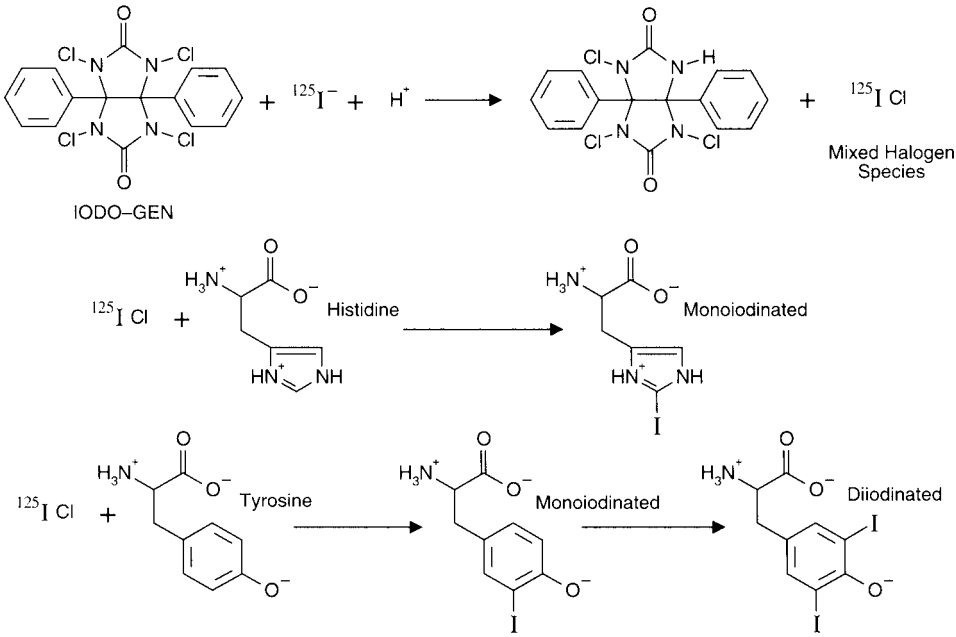


Figure 12.5 IODO-GEN is a water-insoluble oxidizing agent that can react with $^{125}I^-$ to form a highly reactive mixed halogen species, ^{125}ICl. This intermediate can add radioactive iodine atoms to tyrosine or histidine side chain rings.

Since Iodogen is insoluble in aqueous solution and is plated on the surface of the vessel during the iodination reaction, it is possible to stop the reaction simply by removing the aqueous phase. The plating technique is important for the successful use of this reagent. Failure to plate properly the Iodogen reagent on the surface of the reaction vessel may cause the oxidizing agent to become suspended in the reaction medium. However, even with well-plated vessels, there is some potential that a portion of the iodinating reagent can break off in small pieces and contaminate the aqueous phase. For this reason, it is not advisable to stop the iodination reaction only by removing the supernatant as in the case of Iodobeads (Section 2, this chapter). To be certain that the iodination has stopped, an aliquot of sodium metabisulfite can be added to assure complete cessation of the oxidative process. Alternatively, immediate separation of the iodinated protein from the reactants by gel filtration can be used to stop the reaction (any suspended particles of Iodogen will be filtered out on the top of the gel).

Specific radioactivity of 1×10^5 cpm of ^{125}I per microgram of protein easily can be obtained using Iodogen. Iodination efficiencies are typically 60 percent or better and may be controlled by regulating the amount of I^- concentration added to the reaction.

When iodinating intact cells, Iodogen can be used to radiolabel the outer cell surface proteins or be directed more toward the inner membrane areas simply by modulating the reaction conditions. Membrane proteins in hydrophobic regions can be labeled to a greater extent by including a small excess of carrier iodide, using high salt conditions, or by employing detergents to disrupt the membrane integrity. Cell surface hydrophilic proteins may be preferentially labeled

by not including components which increase cell permeability, by the use of carrier-free iodide, and using short reaction times (Markwell and Fox, 1978).

The following protocol describes the use of Iodogen for the radioiodination of proteins and peptides.

Protocol

Caution: Handle all radioactive substances according to the radiation safety regulations instituted at each facility approved to handle such materials. Use adequate precautions to protect personal safety and the environment from contamination. Dispose of radioactive waste by following approved guidelines.

1. In a fume hood, dissolve 10–100 μg of Iodogen (Thermo Fisher) in 100–500 μl of chloroform, methylene chloride, or DMSO. The use of 10 μg of Iodogen per 100 μg of protein or 107 cells to be iodinated will result in good incorporation yields.
2. Add the Iodogen solution to a clean, dry, glass reaction vessel in an amount needed for the quantity of protein to be labeled. Slowly evaporate the solvent in the vessel using a stream of dry nitrogen or other inert gas. Do not use a strong gas jet, since rapid evaporation or turbulence in the solvent solution will cause uneven Iodogen distribution with possible clumping. Do not merely leave the vessel to dry in a hood, since contaminants or moisture may get into the reagent film. If done properly, the plating process should leave a film of Iodogen on the inner surface of the vessel that is difficult to see—looking like a slight clouding of the glass. After solvent evaporation, seal the container with nitrogen and store in a desiccator until needed.
3. Dissolve the protein to be iodinated in a buffer compatible with its known biological stability. Conditions ranging from pH 4.4 to 9 and temperatures from 0°C to 37°C can be used with good results. The amount of protein to be labeled should be contained in a volume of 100 μl or less. The sample buffer should not contain reducing agents, antioxidants, 2-mercaptoethanol, dithiothreitol (DTT), cysteine, glycerol, high detergent concentrations, or anything that may interfere with the iodination reaction or dislodge the plated Iodogen.
4. Rinse the plated reaction vessel once with sample buffer to remove any loose particles of Iodogen that may not be strongly adhered to the surface of the glass.
5. Add carrier-free $Na^{125}I$ to the reaction vessel in a ratio of about 500 μCi per 100 μg protein.
6. React for 10–15 minutes at room temperature. Optimization of the reaction time and the amount of ^{125}I added to the reaction may have to be done to obtain the best radioactivity incorporation and retention of protein activity.
7. Remove the sample from the reaction vessel. This process should terminate the iodination reaction, unless small Iodogen particles break off from the sides of the vessel. To assure safe handling, carrier NaI may be added to the reaction mixture to a final concentration of 1 mM.
8. Remove excess reactants by gel filtration using a desalting resin.

4. Lactoperoxidase-Catalyzed Iodination

An enzyme-catalyzed process also may be used to form reactive iodine species capable of iodinating proteins and other molecules (Marcholonis, 1969; Morrison and Bayse, 1970). The

enzymatic approach utilizes lactoperoxidase in the presence of H_2O_2 to oxidize $^{125}I^-$ to I_2. The iodine thus formed may react with tyrosine or histidine sites within proteins, forming radiolabeled complexes. Unlike the use of chemical oxidants for iodination, the enzymatic reaction is very pH dependent—the optimum being between pH 6 and 7. If H_2O_2 is directly added to the reaction medium, it must be highly pure with no stabilizing agents such as metals, since they inhibit the oxidation process.

An alternative to direct addition of H_2O_2 is to form it *in situ* through the use of a second enzymatic reaction. Enzymobeads (originally from Bio-Rad, but no longer commercially available) used immobilized lactoperoxidase along with immobilized glucose oxidase to create the necessary oxidative environment. The glucose oxidase reaction transformed added glucose in the iodination medium to the required H_2O_2. As it was formed, the lactoperoxidase (coupled in tandem to the same beads) would catalyze the formation of I_2 (Figure 12.6). The immobilized enzymes create an iodination environment that is more oxidatively gentle than direct addition of a soluble chemical oxidant like chloramine-T.

5. Iodinatable Modification and Crosslinking Agents

Radioiodination can be done by an indirect approach that utilizes a radiolabeled crosslinking or modification reagent which is then used to label the target molecule. One advantage of indirect labeling over direct modification of tyrosine or histidine residues in proteins is to be able to control the iodination to occur with functional groups other than just using indigenous amino acids. In addition, the ability to add a radiolabeled modification agent to a molecule can facilitate the radioactive tagging of substances that normally do not have radioiodinatable sites. Another major advantage of using iodinatable modification agents is to eliminate the potential for oxidative damage to sensitive biological molecules, as may occur when an oxidant is used in direct iodination procedures.

For instance, an amine-reactive modification reagent can be radiolabeled and subsequently used to couple with ε- and N-terminal amines on a protein molecule. The protein is not exposed to oxidative conditions, and the level of radiolabeling can be discretely controlled by the molar ratio of modification reagent addition. The use of iodinatable crosslinking reagents can similarly provide radioactive tags incorporated into conjugates at the time of formation. In addition, iodinatable bioconjugation reagents that react with groups such as sulfhydryls, aldehydes, or other functionalities of limited occurrence in proteins or other macromolecules, can be used to direct the point of radiolabeling to areas away from active centers or binding sites, thus better preserving biological activity. Finally, some photoreactive crosslinking agents can be iodinated, used to label a targeting molecule, photolyzed at the point of binding to its target, and the cross-bridge of the resulting complex chemically cleaved, resulting in the transfer the radiolabel to the targeted component (Chapter 5, Section 3). This process can be used to follow the targeted molecule *in vivo* or in cellular systems.

Direct iodination of proteins and other molecules does not provide the range of experimental options available through indirect labeling. The main disadvantage of the indirect labeling process is the additional steps needed to prepare the radiolabeled crosslinker or modification reagent before iodination of the desired molecule. The following sections discuss some of the major indirect iodination methods, including the reagents available for doing such procedures.

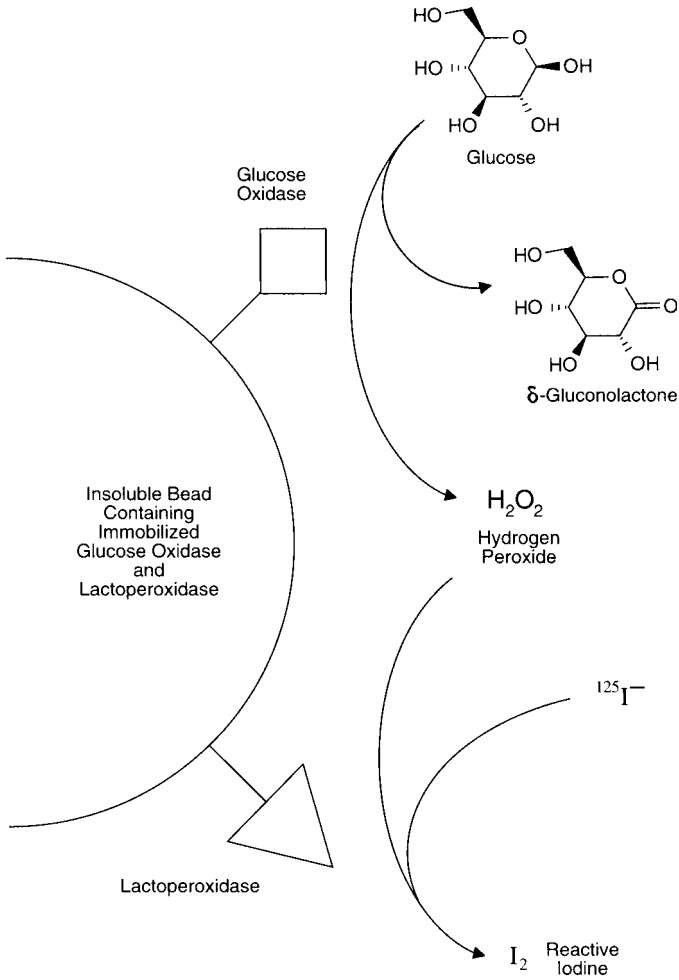
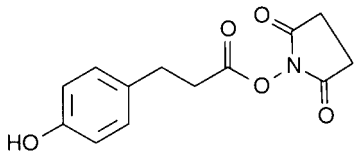


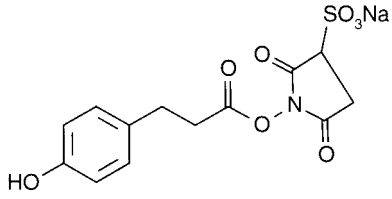
Figure 12.6 The immobilized glucose oxidase/lactoperoxidase system radioiodinates proteins through the intermediate formation of hydrogen peroxide from the oxidation of glucose. H_2O_2 then reacts with iodide anions to form reactive iodine (I_2). This efficiently drives the formation of the highly reactive H_2OI^+ species that is capable of iodinating tyrosine or histidine residues (see Figure 12.2).

5.1. Bolton–Hunter Reagent

Bolton and Hunter (1973) developed the reagent *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (SHPP) for the indirect radioiodination of proteins and other macromolecules (Thermo Fisher). The NHS ester end of the molecule reacts with amine groups in target molecules to form stable amide bond derivatives (Chapter 2, Section 1.4). The other end of the reagent contains a phenolic group that is ideally suited for modification with ^{125}I. Iodination of the phenol group occurs *ortho* to the hydroxyl, thus accommodating either one or two iodine substitutions per molecule (Figure 12.7).



Bolton-Hunter Reagent
N-Succinimidyl-3-
(4-hydroxyphenyl)propionate
MW 263.26



Water-soluble Bolton-Hunter Reagent
N-Sulfosuccinimidyl-3-
(4-hydroxyphenyl)propionate
MW 365.30

The use of the Bolton–Hunter reagent to incorporate radioactivity into proteins results in at least as good incorporation of ^{125}I as direct labeling procedures using an oxidant. In many cases, the degree of radioiodine labeling can be much greater than that possible by direct labeling of tyrosine residues, because the total number of amines in a protein (from N-terminal

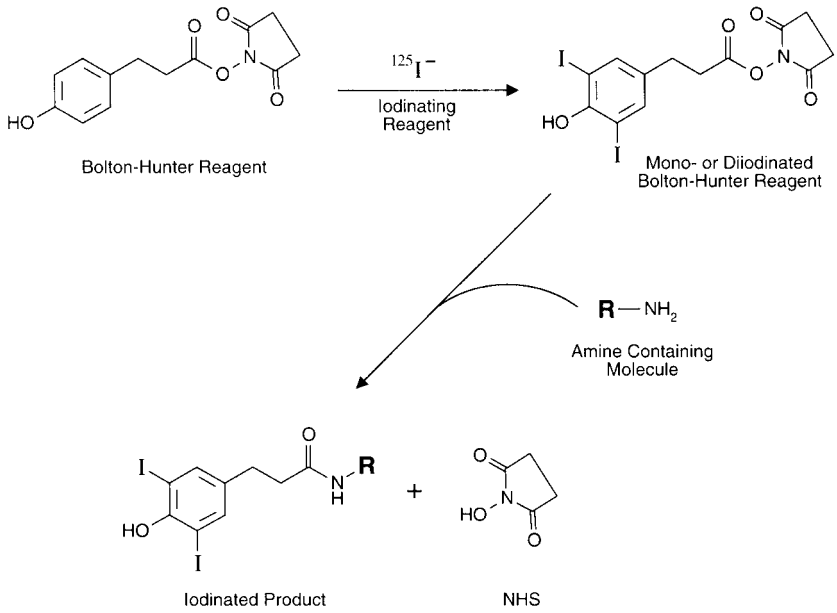


Figure 12.7 The Bolton–Hunter reagent may be radioiodinated at its phenolic ring structure prior to reaction with an amine-containing molecule to form an amide bond modification.

and lysine side chains) is typically significantly more than the number of tyrosines present. The major advantage of the indirect approach, however, is that non-tyrosine containing proteins also may be iodinated. In addition, substances sensitive to oxidant exposure can be labeled without loss of activity or structural degradation. Ultimately, any molecule containing an available amino group can be radioiodinated with SHPP, even if it does not contain a strongly activated aromatic ring system to allow direct iodine substitution. For reviews of protein modification using radiolabeled Bolton–Hunter reagent (see Langone, 1980, 1981; Wilbur, 1992). For its use in labeling cellular components (see Katz *et al.* (1982); Davies and Palek (1981)).

SHPP is relatively insoluble in aqueous environments and must be dissolved in an organic solvent prior to addition to a reaction medium. Suggested solvents include dioxane and DMSO that are low in water content to avoid hydrolysis of the NHS ester.

A water-soluble version of the original Bolton–Hunter reagent also is available, called sulfo-SHPP (Thermo Fisher). This compound contains a negatively charged sulfonate group on the NHS ring structure, which provides enough hydrophilicity to allow direct addition to aqueous reaction mediums.

The following procedure describes the iodination process for the Bolton–Hunter reagent and its subsequent use for the radiolabeling of protein molecules. Modification of other macromolecules can be done using the same general method. For particular labeling applications, optimization of the level of iodine incorporation may have to be done to obtain the best specific radioactivity with retention of biological activity.

Protocol

Caution: Handle all radioactive substances according to the radiation safety regulations instituted at each facility approved to handle such materials. Use adequate precautions to protect personal safety and the environment from contamination. Dispose of radioactive waste by following approved guidelines.

1. Dissolve SHPP (Thermo Fisher) in dry dioxane or DMSO at a concentration of 0.5 mg/ml. Prepare fresh. If sulfo-SHPP is used dissolution in organic solvent is unnecessary, although it may facilitate the addition of a small quantity to an aqueous reaction.
2. Dissolve chloramine-T (Sigma) in 50 mM sodium phosphate, pH 7.5 (reaction buffer) at a concentration of 100 μg per 25–50 μl of buffer. Prepare fresh.
3. Add 2 μl of the SHPP solution to 50 μl of the reaction buffer. Alternatively, dissolve solid sulfo-SHPP into the reaction buffer to give a final concentration of 1 μg/50 μl. To better facilitate measuring out sulfo-SHPP, a more concentrated solution may be prepared in a greater volume and then immediately diluted to this concentration. Once the Bolton–Hunter reagent is in an aqueous environment, the NHS ester end of the compound will hydrolyze. Therefore, all aqueous handling of the reagent from this point on should be done quickly to preserve enough amine-coupling activity to label the protein after the iodination reaction.
4. Immediately add the chloramine-T to the SHPP solution. Mix well.
5. React for 15 seconds with mixing.
6. Add to the iodination reaction 5 μl of DMF and 100 μl of benzene. Mix to extract the iodinated Bolton–Hunter reagent into the organic phase.
7. Remove the aqueous phase and transfer the organic phase into a clean glass vial or tube.

8. Remove the organic solvent by evaporation using a steady, but gentle, stream of nitrogen.
9. Dissolve the equivalent of 250 ng of a protein to be labeled in 2–2.5 µl of ice-cold 50 mM sodium borate, pH 8.5.
10. Add the protein solution to the dried, iodinated Bolton–Hunter reagent.
11. React for 2 hours on ice. An overnight reaction may be done at 4°C.
12. Remove excess labeled Bolton–Hunter reagent by gel filtration or dialysis.

The Bolton–Hunter reagent also may be used to modify a molecule prior to the iodination reaction. In this case, an amine-containing protein or other molecule is coupled via the NHS ester end of the reagent to form an amide bond derivative. This derivative is then iodinated using any of the iodination reagents discussed in this section. This approach can be useful in preparing stable Bolton–Hunter derivatives that can be stored for extended periods until requiring iodination, eliminating the relatively short half-life of ^{125}I-labeled probes.

5.2. Iodinatable Bifunctional Crosslinking Agents

Bifunctional crosslinking agents containing an activated aromatic ring system may be radioiodinated using similar procedures as that described previously for the Bolton–Hunter reagent. Certain conjugation compounds have been designed with the potential for radiolabeling in mind. For instance, there are a number of photoreactive phenyl azide crosslinkers that possess an activating hydroxyl group on their phenyl rings. The phenolic group provides sites of facile iodination ortho and para to the hydroxyl.

The heterobifunctional crosslinker ASBA (4-(*p*-azidosalicylamido)butylamine) (Chapter 5, Section 6.1) is an example of this type of iodinatable photoreactive reagent. The phenyl azide group may be radiolabeled using any standard iodination process (described previously) before coupling of its primary amine end to a carbonyl group on a macromolecule. After allowing the modified molecule to bind to a target, the complex may be photolyzed and the covalent conjugate detected by its radioactivity.

The homobifunctional photoreactive BASED (Chapter 4, Section 5.1) has two photoreactive phenyl azide groups, each of which contains an activating hydroxyl. Radioiodination of this crosslinker can yield one or two iodine atoms on each ring, creating an intensely radioactive compound. Crosslinks formed between two interacting molecules are reversible by disulfide reduction, thus allowing traceability of both components of the conjugate.

An extremely versatile iodinatable heterobifunctional is APDP (*N*-[4-(*p*-Azidosalicylamido) butyl]-3′-(2′-pyridyldithio)propionamide) (Chapter 5, Section 4.2). One end of the crosslinker can couple with sulfhydryl-containing molecules, while the other end is a nonselective photoreactive phenyl azide. Again, the phenyl ring contains an activating hydroxyl group, providing radioiodination capability. Modification of a sulfhydryl-containing molecule may be done after iodination of the reagent. After the labeled molecule is allowed to interact with a target molecule, the photoreactive process can be initiated to form a covalent conjugate. Subsequently, the crosslinks may be cleaved using a disulfide reducing agent, thus transferring the radiolabel to the second molecule.

SASD (sulfosuccinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3'-dithiopropionate) (Chapter 5, Section 3.2) behaves in a similar manner, except it contains an amine-reactive end that can be

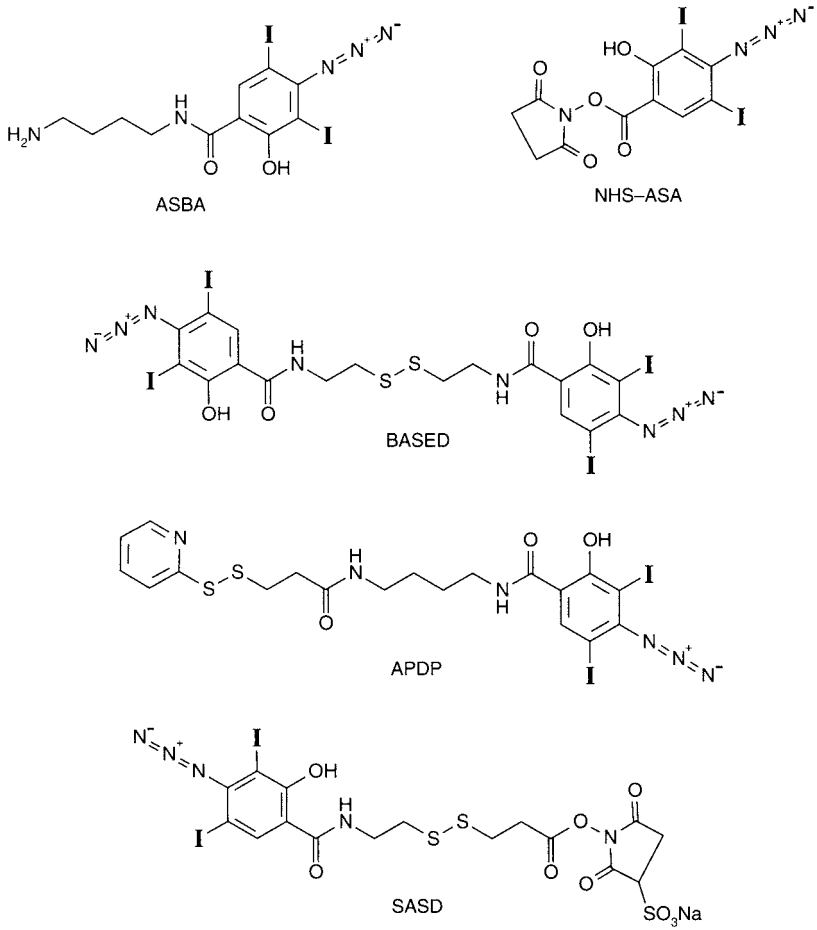


Figure 12.8 Some common crosslinking agents that are capable of being radioiodinated. The sites of iodination are shown in bold.

coupled to proteins and other molecules. Its photoreactive end can be iodinated using any of the radioiodination reagents discussed previously. Just as in the case of APDP, SASD crosslinks can be cleaved by a disulfide reductant to transfer the radioactive component to a second molecule.

Finally, the small amine-reactive and photoreactive crosslinker, NHS–ASA (Chapter 5, Section 3.1), can be iodinated to provide a non-cleavable radioactive conjugate.

Figure 12.8 shows the iodination products resulting from labeling these reagents with ^{125}I. Any of the iodination reagents described previously can be used to radiolabel these compounds prior to their incorporation into target molecules. However, the insoluble iodination reagents are probably the best choice, since the separation of radiolabeled compound from excess oxidant simply involves removing the solution.

There are many other compounds that have been investigated for their use in indirect radiolabeling of proteins. For an excellent overview of these chemical reactions (see Wilbur, 1992).

13

Silane Coupling Agents

A silane compound is a monomeric silicon-based molecule containing four constituents. Since silicon is in the same family as carbon on the periodic table, it too is able to form covalent bonds with four other atoms. However, it is less electronegative than carbon and undergoes reactions that are unique compared to typical organic compounds. Silanes that contain at least one bonded carbon atom are called organosilanes. Organosilanes also can have hydrogen, oxygen, or halogen atoms directly attached to the silicon atom core. Some of these derivatives are highly reactive and can be used to form covalent linkages with other molecules or surfaces. The more useful organosilanes are those that contain a functional organic component in addition to one or more silane reactive groups. The organic portion also may contain a reactive group, which allows conjugation of the organosilane molecule to other organic compounds. Conversely, the silane reactive groups typically are unreactive toward organic molecules, but can covalently couple to certain inorganic substrates. The advantage of this type of functional silane derivative is to promote the bonding of an organic molecule to an inorganic particle, surface, or substrate.

The general structure of a functional silane coupling agent is shown in Figure 13.1. The organic arm typically has a structure that terminates in a functional group or reactive component, which facilitates the covalent linkage to another organic molecule. The other part

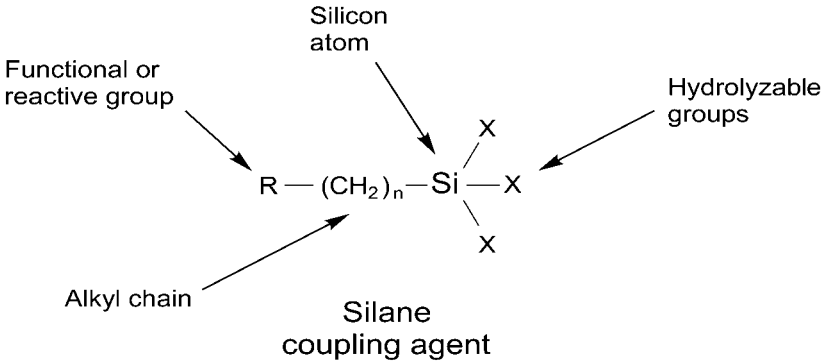


Figure 13.1 The general structure of a silane coupling agent includes a functional group or reactive group at the end of an organic spacer. This alkyl chain is attached to the central silicon atom, which also has up to three hydrolysable groups attached to it.

consists of the silane reactive groups attached directly to the silicon atom and can be of several types. They may comprise simply a hydrogen atom (called a silicon hydride), a halogen-silicon derivative, such as a chlorine atom (called a chlorosilane), an —OH group (called a silanol), or groups containing methyl ether or ethyl ether organic constituents (called a methoxy- or ethoxysilane, respectively).

The general reactions of silane coupling agents toward inorganic substrates are illustrated in Figure 13.2. The reaction mechanism and formation of a reactive intermediate can be different from that usually encountered with organic reactive groups. Unlike most other reactive groups discussed in this book, the most common silane coupling groups (alkoxy) often must first undergo hydrolysis to form a reactive intermediate, which then couples to the substrate. The hydrolysis of an alkoxysilane produces a silanol, which actually is the highly reactive form necessary for coupling to inorganic surface hydroxyls.

The initial reaction that occurs in solution or near an inorganic substrate is condensation of the silane coupling agents together to form a polymer matrix linked together by —Si—O—Si— bonds. Concurrently, the growing silane network interacts with the inorganic substrate through the formation of a hydrogen bonding network with the —OH groups on its surface. Another condensation reaction then occurs, usually requiring heat or vacuum to remove water, which results in the formation of covalently linked organosilane polymer to the surface, forming stable siloxane linkages (or oxane bonds). The organosilane coating applied in this manner does not result in a monolayer, but a thicker polymer layer with the reactive organic components extending out from the surface. The thickness of the organo-siloxane layer is dependent on the concentration of silane coupling agent used in the reaction and the amount of water present in the solution. Gelest reports that deposition of a silane coupling agent using a 0.25 percent aqueous solution will result in a layer appr oximately 3–5 silanes thick.

Alkoxysilanes are used for modification of surfaces due to the instability of silanol derivatives, which will spontaneously hydrogen bond and conjugate together in solution. Forming the alkoxy derivative acts as a stabilizer to prevent silanol polymerization. However, alkoxysilanes typically are unreactive with substrate hydroxyl groups under ambient temperature conditions. Ethoxysilanes are virtually unreactive to substrate —OH groups without prior hydrolysis. Methoxysilanes are the most reactive, but only react very slowly at room temperature. However, under the right conditions, both methoxy- and chlorosilane groups are sufficiently reactive to couple directly with inorganic substrate functionalities without prior hydrolysis. For instance, the addition of a catalyst to the reaction to increase the hydrogen bonding capability of substrate hydroxyls has been done to increase the reaction rate for methoxysilanes (Kanan *et al*., 2002). Also, chlorosilanes and methoxysilanes can be reacted in an organic solvent without the presence of water (i.e., in THF, toluene, or hydrocarbon solvents), if done under refluxing conditions to drive the reaction to completion. In this case, a siloxane polymer network doesn't form in solution, because no hydrolysis occurs to form silanols on the silane coupling agents. Therefore, instead of a thick polymer layer forming on the substrate as in aqueous reactions, a monolayer results where each organosilane is coupled directly to the substrate via a siloxane bond. The reactive organic components stick out from this monolayer for eventual conjugation with biomolecules or other molecules.

Therefore, functional silanes in a sense are bifunctional compounds that can be used to attach one substance to another. They have been used for many years as adhesive agents to promote the bonding of an organic layer to an inorganic layer. Some examples are in the coupling of inorganic metals, particles, fiberglass, or fillers to organic polymers, plastics, rubber,

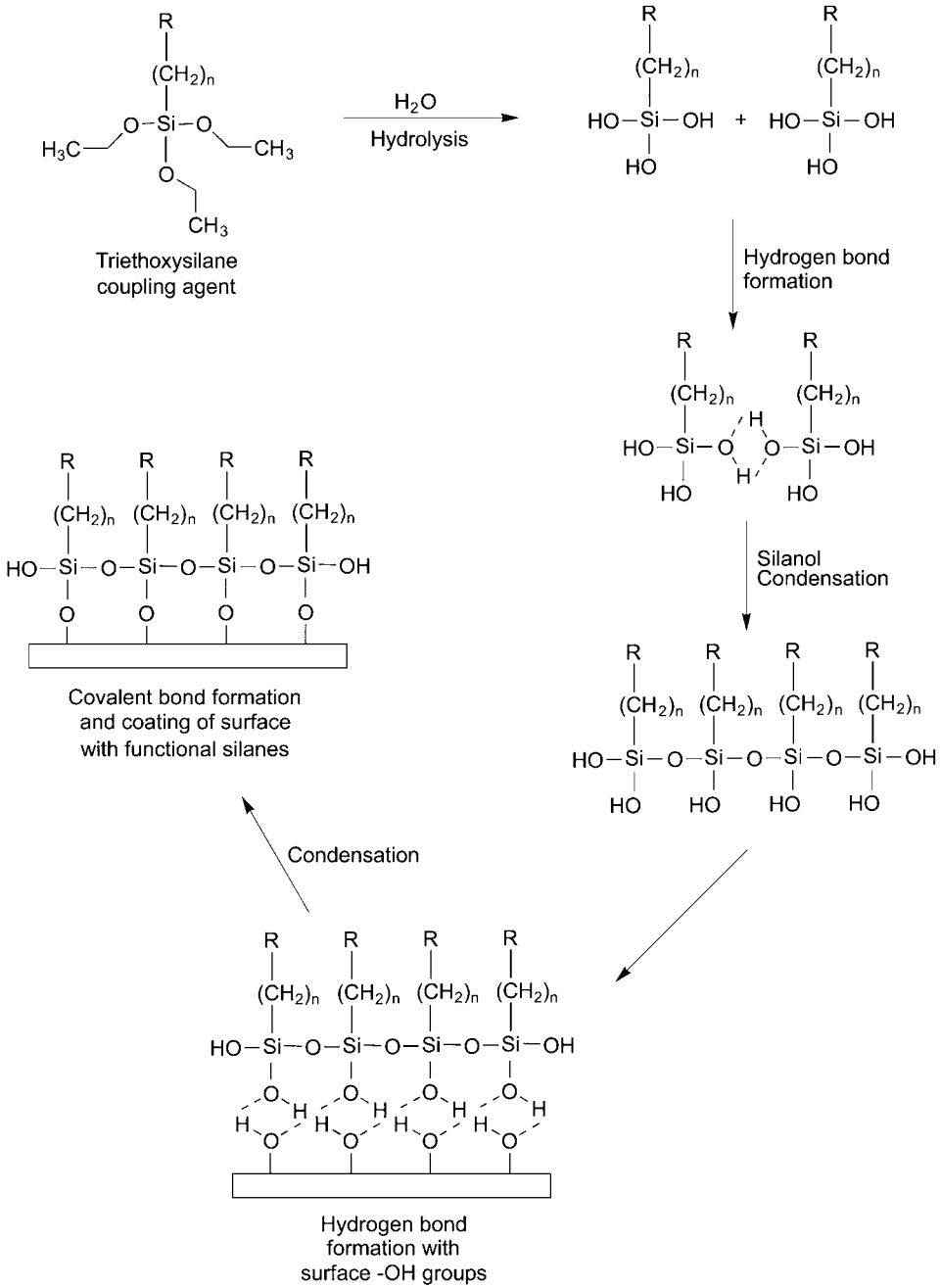


Figure 13.2 The reactions involved with the coupling of an organosilane compound to an inorganic surface containing available —OH groups. The first step in the reaction involves the hydrolysis of the alkoxysilane groups to form highly reactive silanols. The silanols undergo hydrogen bonding with other silanols in solution and on the substrate, resulting in a network of associated organosilane derivatives. A condensation reaction then takes place to form a polymerized coating of the organosilane on the surface of the substrate.

or resins. The uses for functional silanes thus are extensive and span many industries, such as automotive, building materials, coatings, and electronics, only to name a few. For reviews of silane coupling agents and their uses, see Plueddemann, 1991, and VanDerVoort *et al.*, 1996.

Some common inorganic substrates for use with silane coupling agents in approximate order of efficiency and stability for modification include silica, quartz, glass, and the oxides of aluminum, copper, tin, titanium, iron, chromium, zirconium, nickel, and zinc. All of these substrates can have functional inorganic —OH groups on their surface that react with the silanols on the silane coupling agents to form siloxane bonds. Sometimes the surfaces require prior treatment to form the —OH groups and remove contaminants, which will interfere with the silanation process. Glass, for instance, often needs to be treated with acid (5 percent HCl) for several hours to remove non-binding metal ions, especially sodium, potassium, and calcium, which are ubiquitous in the environment. In addition, treatment with a mixture of 25 percent sulfuric acid and 15 percent hydrogen peroxide (piranha solution) for about 30 minutes is done to create a high density of hydroxyl functionalities suitable for silane modification. Glass slides also can be cleaned and washed prior to modification with a silane with DMSO, ethanol, and water, and then etched using 10 percent NaOH (w/w) in water for 1 hour.

The distinctive bifunctional nature of silane coupling agents has led to their application in bioconjugate chemistry. There are many silane coupling agents commercially available that contain functional groups or reactive groups that can be used to covalently link biomolecules to inorganic substrates (Dow Corning, Gelest). Inorganic substrates treated with a suitable silane coupling agent subsequently can be used to couple antibodies, proteins, oligonucleotides, or other biomolecules containing the appropriate chemical groups for reaction. By judicious choice of the proper organo-functional component, a silane coupling agent can be used to design virtually any bioconjugation complex.

1. Silane Reaction Strategies

The reaction techniques that can be used with functional silane coupling agents are varied. Reactions can be done in aqueous solution, entirely in organic solvent, organic solutions containing a small amount of water, and even in the vapor phase. They also can be done at room temperature or under elevated temperature conditions. The choice of reaction strategy often is governed by the type of substrate initially being modified by the silane compound and the inorganic reactive groups on the silane. For instance, if the inorganic substrate can be treated by mixing or suspending it in a solution of functional silane, such as with particle coatings, then this may be simplest option for silanization. Another choice may be to dip the substrate into the silane solution, as is sometimes done with glass slides and other small surfaces that can be handled in trays or baskets. Alternatively, complex surfaces, such as those often encountered with devices, may be treated in a cha mber wherein the silane compound is volatilized by heating or under vacuum. This option usually results in the uniform modification of all surfaces with a thin layer of functionalized silane.

The following sections describe the major organosilane reaction strategies used to modify surfaces and provide suggested protocols, which may be used with the functional silane compounds described later in this chapter. Many organosilane compounds are initially only sparingly soluble in aqueous solution. Therefore, solutions containing silanes in either 100 percent aqueous or in a water/organic mixture containing a large amount of the aqueous component

often will appear as insoluble two-phase systems. As the alkoxy groups hydrolyze, the silanols will dissolve into the aqueous solution and the solution will become clear. However, if the solution is allowed to sit long enough, the silanols will react, form siloxane linkages, and polymerize in solution. At this point, the solution again will become cloudy due to these large polymers.

1.1. Aqueous/Organic Solvent Deposition

This method involves the deposition of functional silane onto an inorganic substrate using a dilute solution of water in organic solvent. The small amount of water at acid pH facilitates the hydrolysis of the alkoxy groups on the silane to form the reactive silanols necessary for coupling. The method of silane solution contact with the substrate may be accomplished by suspension stirring, dipping, spinning, or spraying the silane solution.

Protocol

All operations should be done in a well-ventilated fume hood. Use care not to inhale vapors or get reactive silanes on your skin or in your eyes.

1. Prepare a solution containing 3–5 percent water in ethanol (v/v) and adjust the pH to 4.5–5.5 with acetic acid.
2. Dissolve a silane coupling agent in the acidic water/ethanol solution with stirring to a final concentration of 2–5 percent (v/v). Allow hydrolysis to occur for 5 minutes at room temperature to form reactive silanols.
3. Contact the inorganic substrate with the silane solution for as brief as 2 minutes to as long as several hours, depending on the degree of organosilane polymer deposition desired on the surface. Benters *et al.*, (2002) performed the reaction on glass slides in a stirring bath for 2 hours. Optimization of the reaction time should be done to determine the best performance of the modified substrate in its intended application.
4. Wash the substrate several times with ethanol or the water/ethanol mixture to remove excess silane compound.
5. Cure the organosilane-modified substrate by incubation at 110°C for 30 minutes or at room temperature in a low humidity environment. Curing under vacuum also will aid in the removal of water and the formation of siloxane bonds.

1.2. Aqueous Deposition

Functional organosilanes can be applied to substrates directly from aqueous solutions, provided the silane compound is soluble is water.

Protocol

1. Dissolve an alkoxysilane in water at a concentration of 0.5–2.0 percent (v/v). If the compound is not very soluble in water, a nonionic detergent can be added to the solution at 0.1 percent to promote solubility.

2. Adjust the solution with acetic acid to pH 5.5.
3. Contact the substrate with the silane solution from 2 minutes to as long as 1 hour, depending on the degree of organosilane polymer deposition desired on the surface. Optimization of the reaction time should be done to determine the best performance of the modified substrate in its intended application.
4. Remove excess solution and cure at 110–120°C for 30 minutes to remove traces of water and form the siloxane bonds.
5. Wash the substrate with water or buffer.

1.3. Organic Solvent Deposition

This method of silanation, which uses organic solvent without the addition of water, is suitable for highly reactive silane derivatives, such as chlorosilanes, aminosilanes, and methoxysilanes. This procedure will not work for ethoxysilanes, as these compounds are not reactive enough without prior hydrolysis to create the silanol. This method is convenient to use for silica particle modification and for the functionalization of metallic nanoparticles having the requisite —OH groups present (see Chapter 14, Section 5).

Protocol

1. In a fume hood, dissolve the organosilane coupling agent containing chloro-, amino-, or methoxysilane reactive groups in toluene, THF, or a hydrocarbon solvent at a concentration of 5 percent silane.
2. To assure a thin monolayer of silane modification on the substrate, first remove all traces of water by drying it at 150°C for 4 hours. This step is especially important for nanoparticle modification, as the high surface area of the particle population can hold a lot of water by hydrogen bonding. Note that if the substrate contains water, a thicker polymer layer of silane will build up on it due to hydrolysis of the reactive groups to silanols close to the surface of the substrate. After drying, wash the particles with solvent several times by centrifugation before resuspending them in the silane solution. For other substrates, submerge them in the silane solution with mixing.
3. React with gentle stirring for 12–24 hours by heating to reflux in a fume hood.
4. Cool the reaction and wash the substrate with solvent to remove excess silane reagent and reaction by-products. The modified substrate may be dried or washed into aqueous buffer for further conjugation with biomolecules.

1.4. Vapor Phase Deposition

Many silane coupling agents can be applied to substrates by volatilization in an enclosed chamber under heat or vacuum. In this approach, the substrate is placed within the chamber in a fashion to allow for vapor phase molecules to access all areas that are to be derivatized. This method is commonly used for silanizing glass slides or substrates that are difficult to suspend in a silane solution. Slides are often placed in racks within the chamber and all surfaces get modified

with silane in a uniform manner. Vapor deposition also uses a very small amount of organosilane compound compared to what may be necessary to immerse fully a substrate device in solution.

Protocol

1. In an enclosed chamber made of glass or acrylic, such as a vacuum chamber, place the substrate to be modified in such a manner as to expose the surfaces to the vapor phase.
2. Place the organosilane coupling agent in a small reservoir under or next to the substrate in the chamber. If volatilization by heating is to be done, an explosion-proof heating device should be used to maintain the silane solution temperature at 50°C or above. Alternatively, apply a vacuum to the chamber until the silane compound begins to volatilize. The vacuum tubing may be clamped off to maintain vacuum within the chamber once a sufficient level of evacuation has been reached. Vapor phase deposition usually requires that the silane have at least a 5 mm vapor pressure to achieve adequate concentration in the atmosphere within the chamber.
3. React for 4–24 hours within the chamber to result in a uniform coating of the substrate surface with organosilane. Often surfaces are coated overnight to complete the reaction.

2. Functional Silane Compounds

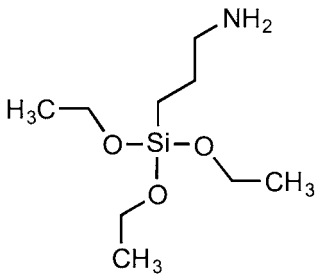
Functional silane compounds containing an organo-functional or organo-reactive arm can be used to conjugate biomolecules to inorganic substrates. The appropriate selection of the functional or reactive group for a particular application can allow the attachment of proteins, oligonucleotides, whole cells, organelles, or even tissue sections to substrates. The organosilanes used for these applications include functional or reactive groups such as hydroxyl, amino, aldehyde, epoxy, carboxylate, thiol, and even alkyl groups to bind molecules through hydrophobic interactions.

The following sections discuss the organosilane compounds commonly used for conjugation to inorganic surfaces. These reagents and many other silane derivatives are available from a number of commercial sources, which include Dow Corning, Gelest, Aldrich, and others.

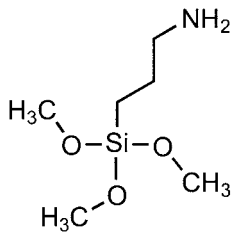
2.1. 3-Aminopropyltriethoxysilane and 3-Aminopropyltrimethoxysilane

These two silane coupling agents are among the most popular choices for creating a functional group on an inorganic surface or particle. Both reagents contain a short organic 3-amino propyl group, which terminates in a primary amine. The only difference in these compounds is the silane reactive portion that contains either a triethoxy group or a trimethoxy group. Thus, the reagents display differences in reactivity toward substrate —OH groups related to the relative reactivity of the alkoxysilane functionalities. The trimethoxy compound is more reactive and can be deposited on a substrate using 100 percent organic solvent without the presence of water to promote hydrolysis of the alkoxy groups prior to coupling. In this case, the organic solvent deposition protocol described in the previous section can be used to modify covalently substrates with a layer of aminosilane. The advantage of this process is that a thinner, more

controlled deposition of the silane can be made to create a monolayer of aminopropyl groups on the surface.



APTS;
3-Aminopropyltriethoxysilane
MW 221.37



3-Aminopropyltrimethoxysilane
MW 179.29

When using 3-Aminopropyltriethoxysilane (APTS) (the triethoxy version), the reaction must occur in at least a partially aqueous environment. The ethoxy groups are not reactive enough to couple spontaneously with the —OH groups on an inorganic surface without prior hydrolysis to form silanols. This is typically done in 5 percent water in ethanol that has been acidified with acetic acid to pH 4.5–5.5. The protocol described in the previous section for aqueous solvent deposition can be used with success to modify surfaces or particles with this reagent (Figure 13.3). This process results in a layer containing about 3–8 organosilanes in thickness, which effectively masks the underlying inorganic substrate with aminopropyl functionalities.

Once deposited on a substrate, the alkoxy groups form a covalent polymer coating with the primary amine groups sticking off the surface and available for subsequent conjugation. Carboxyl- or aldehyde-containing ligands may be directly coupled to the aminopropyl groups using a carbodiimide reaction (Chapter 3, Section 1) or reductive amination, respectively (Chapter 3, Section 4). Alternatively, surfaces initially derivatized with an aminopropylsilane compound can be modified further with spacer arms or crosslinkers to create reactive groups for coupling affinity ligands or biomolecules. For instance, the amine groups may be derivatized with an NHS–PEG_n-azide compound for use in click chemistry or Staudinger ligation reactions for linking proteins or other biomolecules (Chapter 17, Section 4 and 5). This modification forms extremely hydrophilic PEG spacers on the surface that terminate in alkyl azide groups for conjugation with alkyne or phosphine derivatives (Figure 13.4).

Other crosslinking agents that contain an amine-reactive group on one end also may be used to modify and activate the APTS-modified substrate. Surfaces may be designed to contain, for instance, reactive hydrazine groups for conjugation with carbonyl-containing molecules, such as aldehydes formed through periodate oxidation of carbohydrates or natively present at the reducing end of sugars and glycans (Chapter 17, Section 2; Chapter 1, Section 4.6).

Benters *et al.* (2002) used two approaches to modify APTS surfaces. In one instance, the amine groups were acylated using glutaric anhydride to create carboxylate functionalities, which were then activated with NHS/DCC to form the NHS ester. This derivative could be

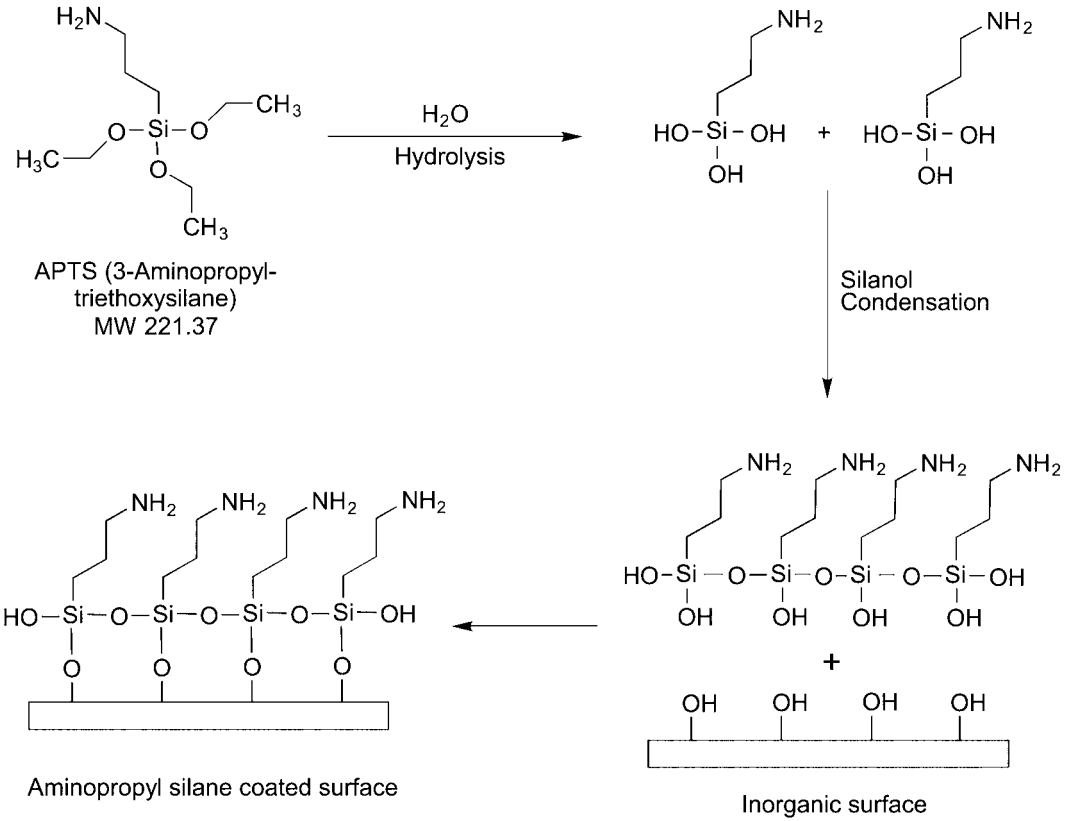


Figure 13.3 The deposition of APTS on inorganic substrates results in the formation of a covalent coating containing primary amine groups.

used to couple amine-containing proteins and other molecules via amide bond formation. In a second activation strategy, the aminopropyl groups on the surface were activated with 1,4-phenylenediisothiocyanate (PDITC) to create terminal isothiocyanate groups for coupling amines. Both methods resulted in the successful coupling of amine-dendrimers to silica surfaces for use in arrays (see Chapter 7, Section 4).

Amine-surfaces prepared using an aminosilane compound can be modified to contain carboxylate groups using the following protocol involving the reaction with anhydride (Figure 13.5). The carboxylates then can be used to couple amine-containing molecules using a carbodiimide reaction with EDC plus sulfo-NHS (Chapter 3, Section 1.2).

Protocol

1. Dissolve glutaric anhydride (Chapter 1, Section 4.2) in DMF at near saturation (use a fume hood). Add triethylamine to a concentration of at least 1 mg/ml to function as a proton acceptor (base).

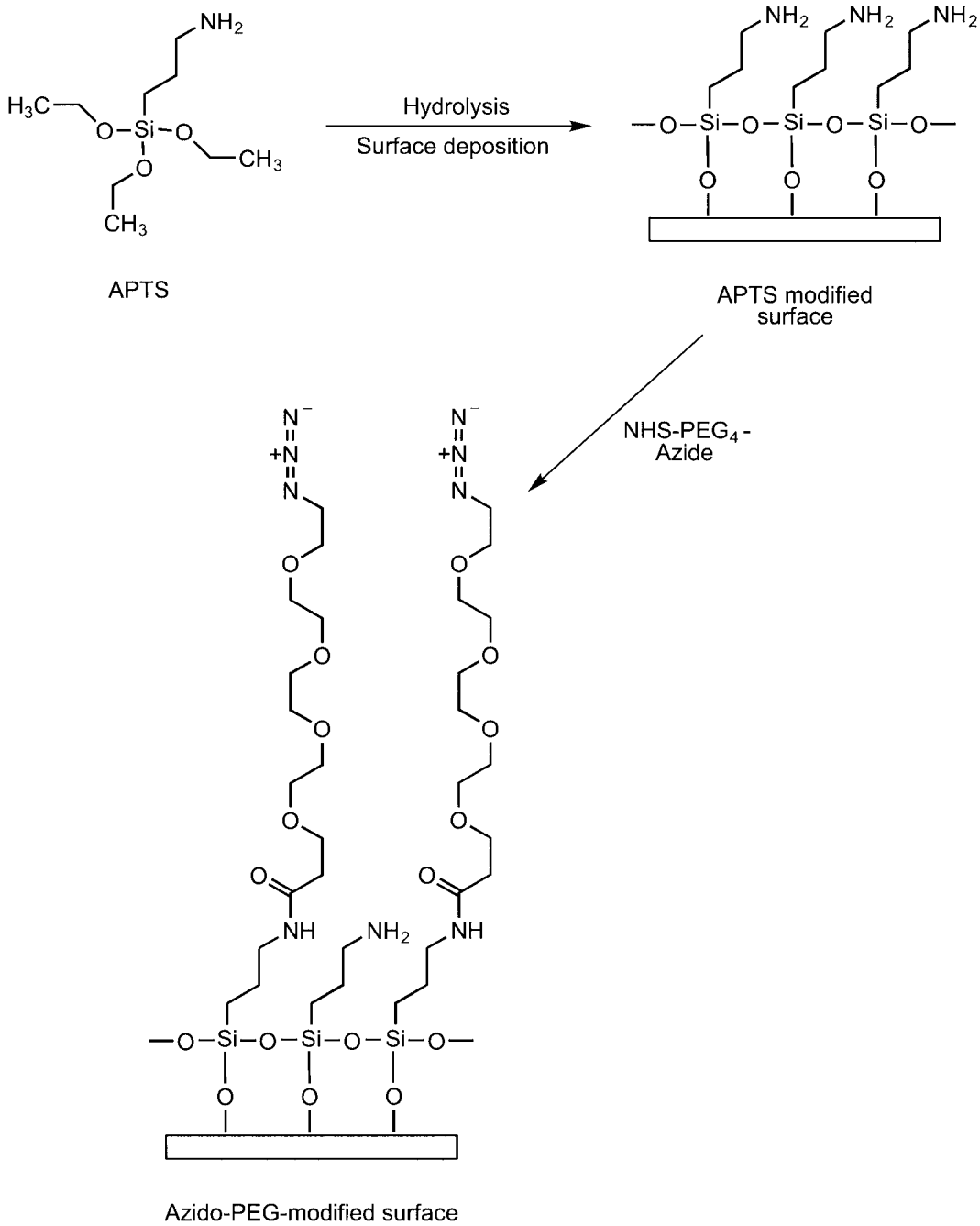


Figure 13.4 APTS-modified surfaces may be further derivatized with amine-reactive crosslinkers to create additional surface characteristics and reactivity. Modification with NHS–PEG_4-azide forms a hydrophilic PEG spacer terminating in an azido group that can be used in a click chemistry or Staudinger ligation reaction to couple other molecules.

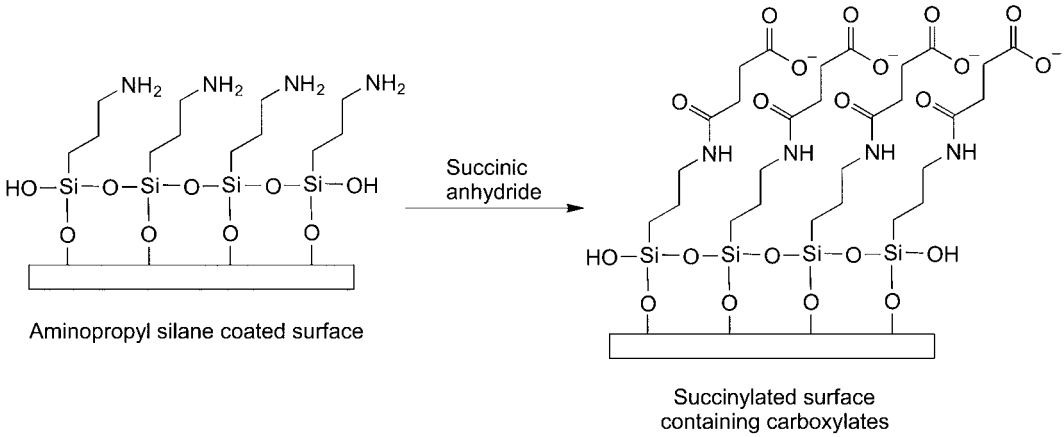


Figure 13.5 Modification of an APTS surface with glutaric anhydride creates terminal carboxylates for coupling of amine-containing ligands.

2. Apply the anhydride solution to the aminosilane-modified surface (typically done by emersion) and mix by stirring.
3. React 2–4 hours at room temperature.
4. Wash the carboxylated surface with DMF and then with water. Dried surfaces are stable indefinitely.

The carboxyl groups on the surface may be activated to an NHS ester for coupling with amine-containing biomolecules according to the following protocol, based on the method of Benters *et al.*, 2002.

Protocol

1. In a fume hood, dissolve *N*-hydroxysuccinimide (NHS) in DMF (highly pure and dried over molecular sieves) at a concentration of 115 mg/ml (1 M) along with 206 mg/ml (1 M) *N*,*N*9-dicyclohexylcarbodiimide (DCC; Chapter 3, Section 1.4).
2. Add the NHS/DCC solution to the carboxylated substrate to immerse fully the surface. Mix by stirring.
3. React for 1–2 hours at room temperature.
4. Wash the surfaces with DMF. The NHS-activated surface may be used to couple amine-containing molecules in a buffer at physiological pH (7.2–7.4) using 50 mM sodium phosphate.

Aminosilane surfaces also may be activated by use of a bifunctional crosslinker to contain reactive groups for subsequent coupling to biomolecules. In one such reaction, *N*,*N*′-disuccinimidyl carbonate (DSC) was used to react with the amines on a slide surface and create terminal NHS-carbonate groups, which then could be coupled to amine-containing molecules (Niemeyer, 2004) (Figure 13.6). The following procedure is based on this method.

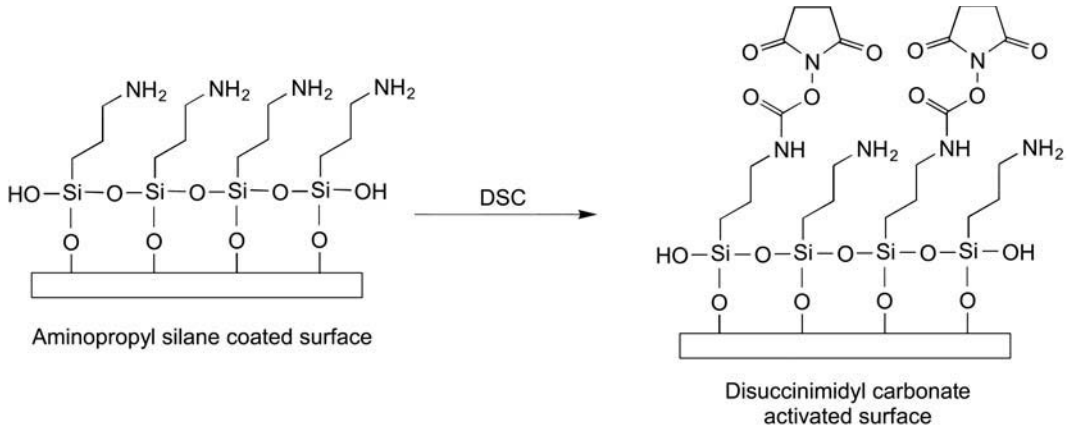


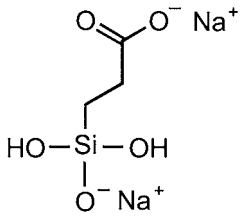
Figure 13.6 APTS-modified surfaces can be activated with DSC to form amine-reactive succinimidyl carbonates for coupling proteins or other amine-containing molecules.

Protocol

1. Dissolve 1.5 m of DSC (Chapter 4, Section 1.7) and 5 ml of diisopropylethylamine (DIEA) in 145 ml of dry acetone (in a fume hood).
2. Add the DSC solution to the aminosilane-slides (or other aminosilane-modified surface) to immerse fully the substrate in the solution and mix by stirring.
3. React for 2 hours at room temperature.
4. Wash the modified surfaces with dry acetone to remove excess reactants and reaction by-products. Dry under nitrogen. The NHS-carbonate groups on the surface are stable to storage under desiccated conditions (package under nitrogen with a desiccant).
5. Amine-containing molecules, such as proteins or amine-modified oligonucleotides, are coupled to the NHS-carbonate activated surface by dissolving them in 50 mM sodium phosphate, pH 7.4, at a concentration of at least 1–10 mg/ml (for proteins). Spotting of biomolecules onto the activated surface may be done to create an array. Since this process usually is done using only microliter quantities or less of solution per spot, the activated surface should be kept in a humidity chamber to prevent evaporation during the coupling reaction.
6. React for 2–4 hours at room temperature.
7. Wash the slides with coupling buffer to remove excess reactants.

2.2. Carboxyethylsilanetriol

Silane coupling agents containing carboxylate groups may be used to functionalize a surface with carboxylic acids for subsequent conjugation with amine-containing molecules. Carboxyethylsilanetriol contains an acetate organo group on a silanetriol inorganic reactive end (Gelest). The silanetriol component is reactive immediately with inorganic —OH substrates without prior hydrolysis of alkoxy groups, as in the case with most other silanation reagents (Figure 13.7). This compound is supplied in a 25 percent aqueous solution as the monosodium salt, and it can be used just by diluting into water or buffer.



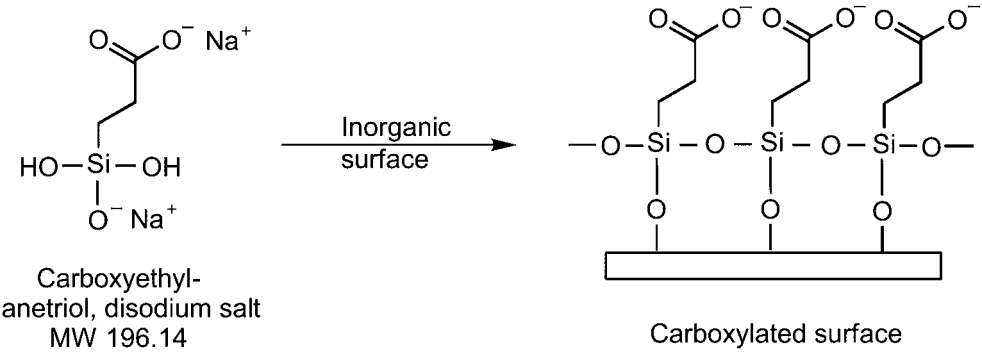
Carboxyethyl-
silanetriol, disodium salt
MW 196.14

Carboxyethylsilanetriol has been used to add carboxylate groups to fluorescent silica nanoparticles to couple antibodies for multiplexed bacteria monitoring (Wang *et al.*, 2007) (see Chapter 14, Section 5). This reagent can be used in similar fashion to add carboxylate functionality to many inorganic or metallic nano-materials, which also will create negative charge repulsion to maintain particle dispersion in aqueous solutions. Covalent coupling to the carboxylated surface then can be done by activation of the carboxylic acid groups with a carbodiimide to facilitate direct reaction with amine-containing molecules or to form intermediate NHS esters (Chapter 3, Section 1).

The following protocol for using carboxyethylsilanetriol is based on the method of Wang *et al.*, 2007. Other metallic particles or surfaces may be modified with this silane compound in like manner.

Protocol

1. Suspend 10 mg of silica particles in 1 ml of 10 mM sodium phosphate, pH 7.4, with gentle mixing.
2. Add 20 µl of the carboxyethylsilanetriol (as the 25 percent aqueous solution) to the particle suspension with mixing.



Carboxyethyl-
silanetriol, disodium salt
MW 196.14

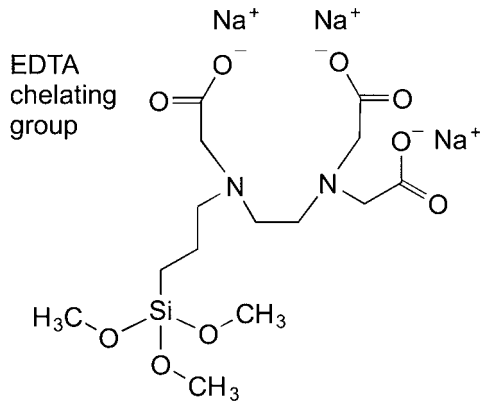
Carboxylated surface

Figure 13.7 Carboxylethylsilanetriol can be used to modify an inorganic substrate to containing carboxylate groups for coupling amine-containing ligands.

3. React at room temperature for 3–4 hours with mixing.
4. Wash the modified particles several times with buffer using centrifugation for separation. A final wash with 10 mM MES, pH 5.5, is done to prepare the sample for coupling amine-containing molecules using a carbodiimide reaction. For suggested protocols, see Chapter 14, Section 4.3, Coupling to Carboxylate Particles.

2.3. *N*-(Trimethoxysilylpropyl)ethylenediamine triacetic acid

Another useful silanation reagent containing carboxylates actually is an effective chelator of metal ions. *N*-(Trimethoxysilylpropyl)ethylenediamine triacetic acid (TMS-EDTA) contains a trimethoxy group for coupling to —OH groups on inorganic substrates and an EDTA group for coordinating metals (minus one carboxylate, which instead functions as the hydrocarbon arm attached to the silicon atom). This compound can be used to coat substrates and provide a metal-chelating functional group for use in affinity separations.



TMS-EDTA;
N-(Trimethoxysilylpropyl)ethylene-
diamine triacetic acid, trisodium salt
MW 462.41

Immobilized metal affinity chromatography (IMAC) has been used for decades as an affinity method for targeting certain functional groups in proteins or other biomolecules (for reviews see Porath, 1992; Hermanson *et al.*, 1992; Winzerling *et al.*, 1992; Lopatin and Varlamov, 1995; Hage, 1999). Various metal ions can be chelated by EDTA affinity groups to provide directed targeting of biological groups such as phosphate modifications or histidine-rich areas in proteins. Thus, phosphorylation sites in proteins can be bound using a silane-EDTA modified surface containing, for instance, gallium (Posewitz and Tempst, 1999). Alternatively, if the chelating groups are charged with nickel or cobalt, the binding of His-tagged proteins can be done.

The preparation of particles or surfaces that are able to capture specifically a fraction of he proteome using metal affinity separations makes possible analysis of distinct protein

populations by mass spec (Zhou *et al.*, 2000). The reactions and use of TMS-EDTA in modifying inorganic surfaces and coordinating metals for affinity chromatography is shown in Figure 13.8.

The coating of surfaces with TMS-EDTA can be done using the general protocol described previously in this chapter entitled Aqueous/Organic Solvent Deposition. The acidified water/organic solvent environment hydrolyzes the methoxy groups to silanols, which then polymerize and covalently link to inorganic surface —OH groups. After a surface has been modified with a chelating ligand, it is washed thoroughly with metal-free water or buffer. Finally, an aqueous solution of the appropriate metal salt is contacted with the surface and the EDTA groups will bind the metal for use in affinity separations.

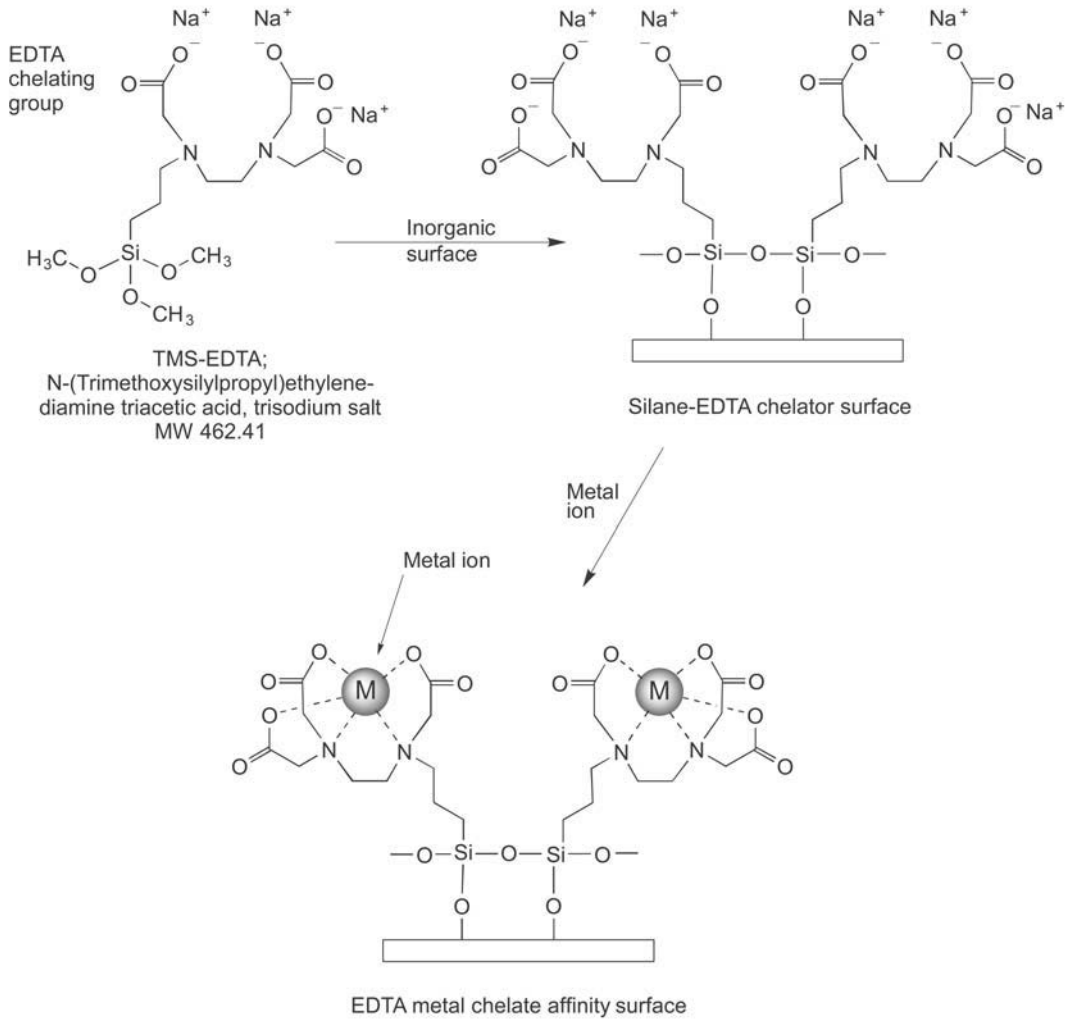
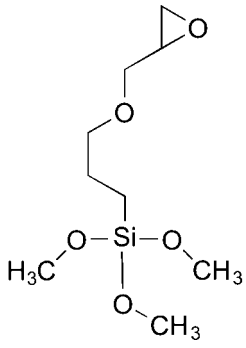


Figure 13.8 TMS-EDTA can be used to modify an inorganic substrate to containing EDTA chelating groups for complexation with metal ions.

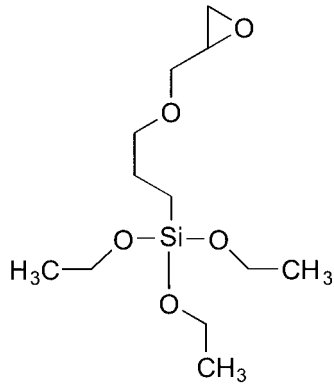
It should be noted that the chelator group in TMS-EDTA is a pentadentate ligand, which will coordinate five bonds with an associated metal ion. Common IMAC ligands use tridentate or tetradentate ligands that have a greater number of available coordination sites for interaction with target molecules. Iminodiacetic acid-based chelators interact with metal ions through three bonds, typically leaving three coordination sites left for affinity binding. Nitrilotriacetic acid-lysine (NTA) chelating groups interact with metal ions through four coordination bonds, leaving two sites available for affinity interactions. However, the TMS-EDTA ligand holds metal ions through five coordination bonds, thus typically having only one remaining for interacting with other molecules. This property may result in lower affinities with biological molecules and change the capture behavior for specific metal interaction sites on proteins. Careful testing should be done for each application envisioned for this metal-chelating silane coupling agent.

2.4. 3-Glycidoxypropyltrimethoxysilane and 3-Glycidoxypropyltriethoxysilane

Two very useful silane modification agents are glycidoxy compounds containing reactive epoxy groups. Surfaces covalently coated with these silane coupling agents can be used to conjugate thiol-, amine-, or hydroxyl-containing ligands, depending on the pH of the reaction (Chapter 2, Section 4.1). Thus, 3-glycidoxypropyltrimethoxysilane (GOPTS) or 3-glycidoxypropyltriethoxysilane can be used to link inorganic silica or other metallic surfaces containing —OH groups with biological molecules containing any three of these major functional groups. GOPTS has been used most often in bioconjugation applications, but the triethoxysilane compound also may be used in similar protocols.(Figure 13.9)



GOPTS;
3-Glycidoxypropyl-
trimethoxysilane
MW 236.34



3-Glycidoxypropyl-
triethoxysilane
MW 278.42

The reaction of the epoxide with a thiol group yields a thioether linkage, whereas reaction with a hydroxyl gives an ether and reaction with an amine results in a secondary amine bond. The relative reactivity of an epoxy group is thiol > amine > hydroxyl, and this is reflected by

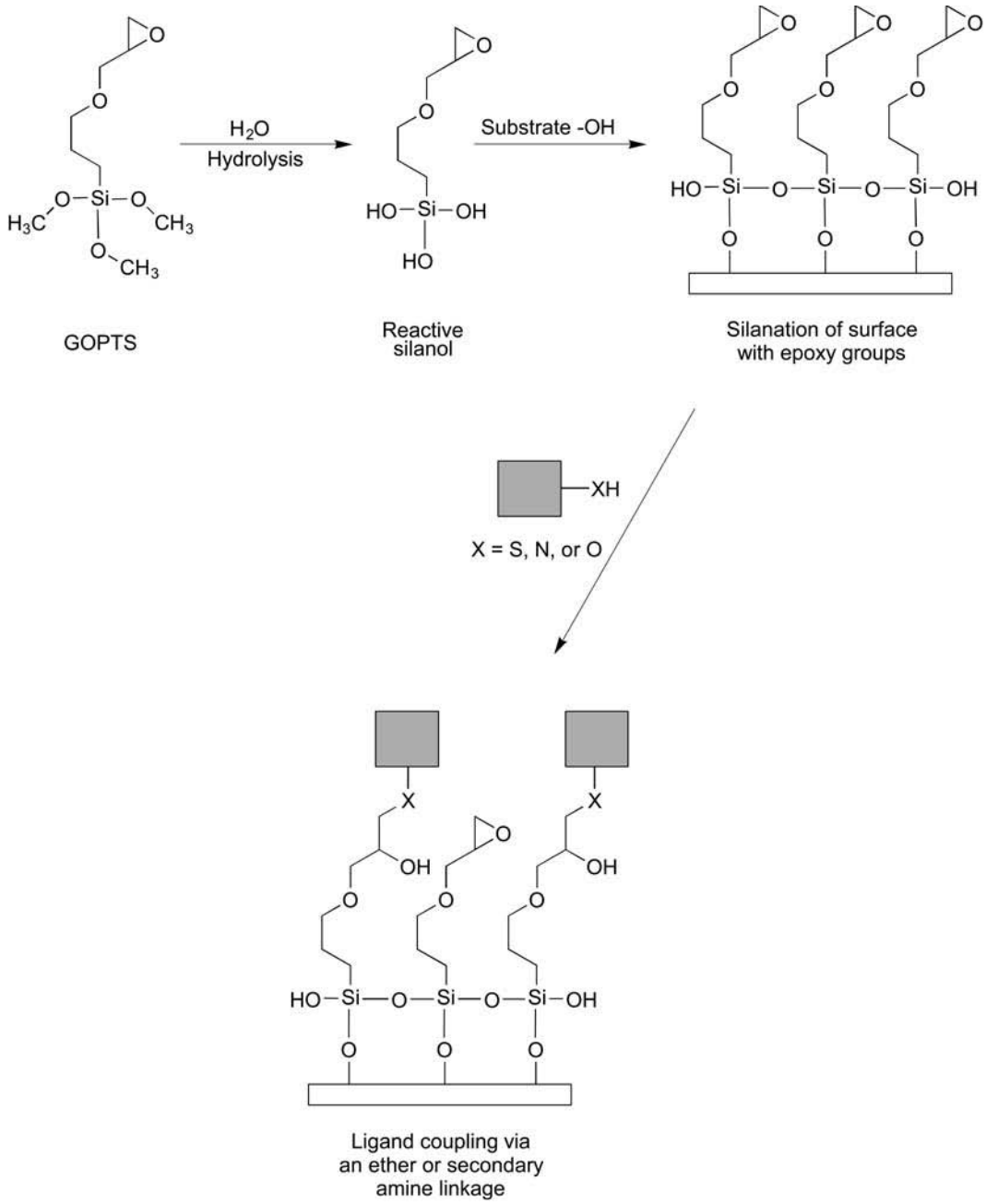


Figure 13.9 Epoxy-containing silane coupling agents form reactive surfaces that can be used to couple amine-, thiol-, or hydroxyl-containing ligands.

the optimal pH range for each reaction. In this case, the lower the reactivity of the functional group the higher the pH required to drive the reaction efficiently.

GOPTS has been used to create a high-density PEG surface on glass slides for use in arrays (Piehler *et al.*, 2000). It also has been used in the development of a high-throughput analyzer using biochip technology on aluminum oxide sheets (FitzGerald *et al.*, 2005) and in the activation of glass surfaces for detection of antibodies specific for hepatitis B and C viruses (Duan *et al.*, 2005).

The following protocol is based on these methods. All operations should be done in a fume hood, including wearing proper protective clothing.

Protocol

1. Prepare glass slides by washing with acid (5 percent HCl) for several hours to remove non-binding metal ions, especially sodium, potassium, and calcium. Treatment with a mixture of 25 percent sulfuric acid and 15 percent hydrogen peroxide (piranha solution) for about 30 minutes is done to create a high density of hydroxyl functionalities suitable for silane modification. Glass slides also can be cleaned and washed prior to modification with a silane with DMSO, ethanol, and water, and then etched using 10 percent NaOH (w/w) in water for 1 hour.
2. Prepare a GOPTS solution in o-xylene or 95 percent ethanol at a concentration of 2 percent (v/v). If the organic solvent is used, add 2 mg/ml N-ethyldiisopropylamine (DIPEA) as base.
3. Immerse the glass slides in the GOPTS solution and mix by stirring.
4. React at 37°C (for the ethanol solution) or 55°C (for the *o*-xylene solution) for at least 5–6 hours with mixing.
5. Wash slides thoroughly with solvent and then dry in an oven at 135°C for 1 hour (explosion-proof oven). The slides are now ready to couple ligands through their epoxy groups.

For protocol suggestions on conjugation to epoxy groups, see Chapter 2, Sections 1.7 and 4.1. Also, see Chapter 14, Section 4.11, Coupling to Epoxy Particles, for a method to attach affinity ligands to surfaces that are activated with epoxide groups.

2.5. Isocyanatopropyltriethoxysilane

Isocyanate groups are extremely reactive toward nucleophiles and will hydrolyze rapidly in aqueous solution (Chapter 2, Sections 1.2 and 4.7). They especially are useful for covalent coupling to hydroxyl groups under non-aqueous conditions, which is appropriate for conjugation to many carbohydrate ligands. Isocyanatopropyltriethoxysilane (ICPTES) contains an isocyanate group at the end of a short propyl spacer, which is connected to the triethoxysilane group useful for attachment to inorganic substrates. Silanation can be accomplished in dry organic solvent to form reactive surfaces while preserving the activity of the isocyanates.

An isocyanate reacts with amines to form isourea linkages and with hydroxyls to form carbamate (urethane) bonds. Both reactions can take place in organic solvent to conjugate molecules to inorganic substrates (Figure 13.10). The solvent used for this reaction must be of high purity and should be dried using molecular sieves prior to adding the silane compound.

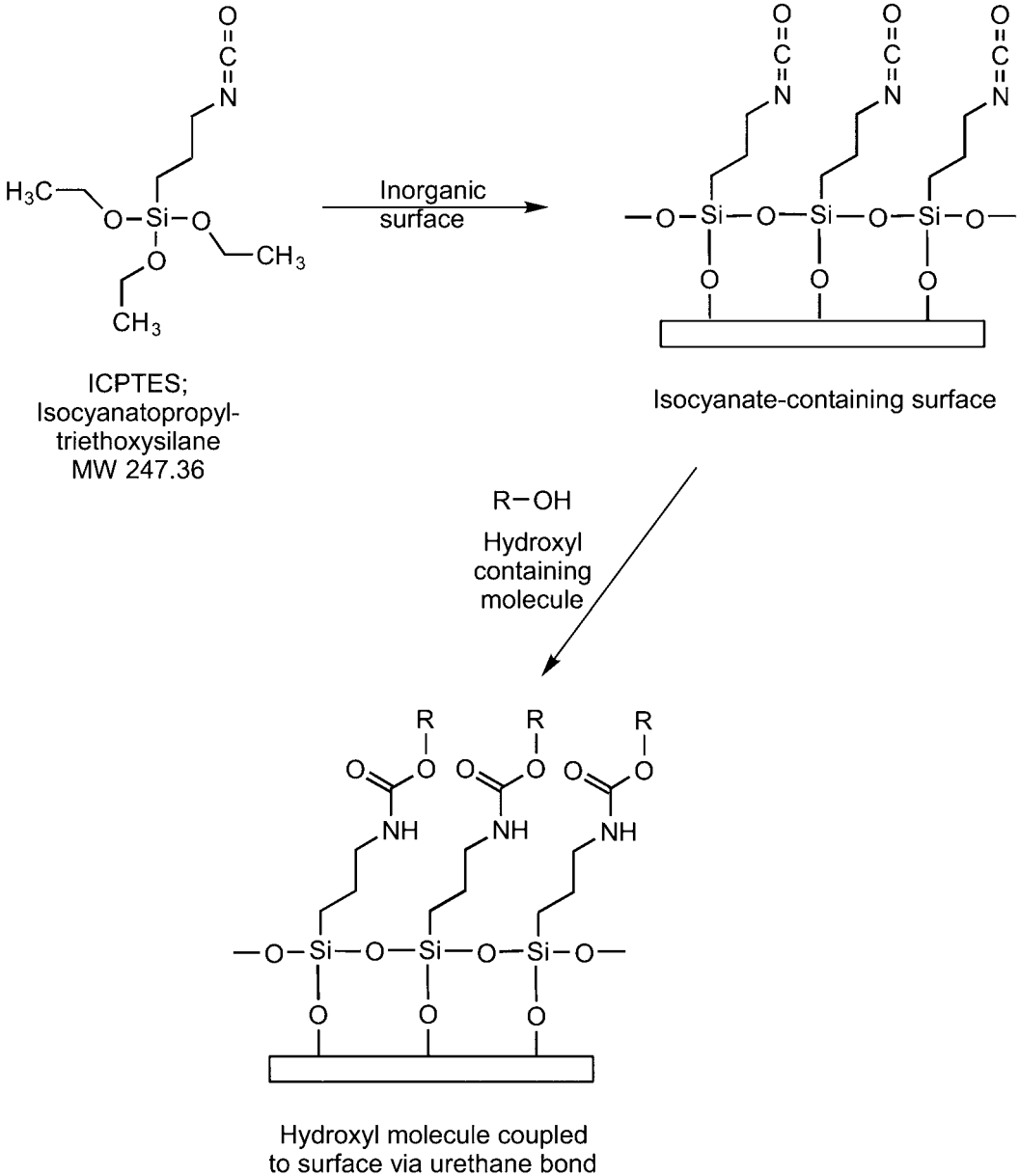
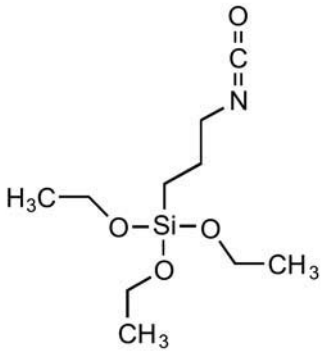


Figure 13.10 The isocyanate-containing silane coupling agent can be used to couple hydroxyl-containing molecules to inorganic surfaces. The reactions should be done in dry organic solvent to prevent hydrolysis of the reactive group.



ICPTES;
Isocyanatopropyl-
triethoxysilane
MW 247.36

Silva *et al.* (2005) used ICPTES to create novel chitosan-siloxane hybrid polymers by coupling the isocyanate groups to the functional groups of the carbohydrate and forming a silica polymer using the triethoxysilane backbone. Boev *et al.* (2005) used this functional silane coupling agent to prepare CdS fluorescent nanoparticles in a urea-silicate matrix. ICPTES and APTS have been have been used in combination to create organically modified silica xerogels through carboxylic acid solvolysis, which formed hybrid materials that have luminescent properties (Fu *et al.*, 2006).

The following protocol is based on the method of FitzGerald *et al.* (2005), who used the technology to develop aluminum oxide-based biochips for high-throughput analysis.

Protocol

1. In a fume hood, dissolve ICPTES in anhydrous toluene at a concentration of 5 percent (v/v) and also containing 1 percent DIPEA base.
2. Clean and prepare an inorganic substrate to remove unreactive metal salts and to create —OH sites for coupling the silane compound. For glass, this can be done using acid or base washes. Formation of —OH groups on glass typically is done using piranha solution, which is 25 percent sulfuric acid and 15 percent hydrogen peroxide. For cleaning aluminum oxide sheets, use an alkaline detergent solution and apply sonication for 1 hour, then wash with water.
3. Treat the inorganic substrate with the ICPTES solution by immersion and react at 50°C overnight with mixing.
4. Wash the derivatized substrate with toluene at least twice and then rinse a final time with acetone.
5. Dry the modified substrate at 120°C for 30 minutes in an explosion-proof oven.

Many other functional silane coupling agents are available from commercial suppliers, including hydroxyl, aldehyde, acrylate and methacrylate, and anhydride compounds. Substrate modification procedures similar to those discussed above can be used with these reagents to link a biomolecule to an inorganic surface or particle.

14

Microparticles and Nanoparticles

The use of particles in biological assays and other applications dates back many decades (Singer and Plotz, 1956). Assays and detection systems made by coupling affinity ligands to particles of nanometer or micrometer diameter have been used in diagnostic tests and numerous other research procedures. Latex microspheres and gold nanoparticles perhaps were the earliest examples of solid phase spheres used for these purposes. With the recent explosion in nanotechnology, the relevant particle size has shrunk by 2–3 orders of magnitude and the applications for particles have dramatically expanded. Nanoparticles and microparticles are used in agglutination tests and assays, particle capture ELISA methods, lateral flow tests, solid phase assays, scintillation proximity assays, polymerase chain reaction (PCR) tests, superparamagnetic-based assays and magnetic separation systems, biosensors, as enhancers of Raman spectral signals, in light scattering assays, and as fluorescent labels or stains for detecting biological molecules.

1. Particle Types

The types of particles used in biological applications are extremely varied. Most often, they are nonporous in nature and spherical in shape. However, the material science revolution in nanotechnology has provided particle types and compositions of almost limitless shape and size, including spherical, amorphous, or aggregate particles, as well as elaborate geometric shapes like rods, tubes, cubes, triangles, and cones. In addition, new symmetrical organic constructs have emerged in the nanometer range that include fullerenes (e.g., buckyballs), carbon nanotubes, and dendrimers, which are highly defined synthetic structures used as bioconjugation scaffolds in various applications (these specialized nanoparticles are discussed in Chapter 7; Chapter 9, Section 10; and Chapter 15).

The chemical composition of particles can be just as varied as their shape. Commercial particles can consist of polymers or copolymers, inorganic constructs, metals and semiconductors, superparamagnetic composites, biodegradable constructs, and synthetic dendrimers and dendrons. Often, both the composition of a particle and its shape govern its suitability for a particular purpose. For instance, composite particles containing superparamagnetic iron oxide typically are used for small-scale affinity separations, especially for cell separations followed by flow cytometry analysis or fluorescence-activated cell sorting (FACS). Core–shell semiconductor particles, by

contrast, are the basis for quantum dot nanoparticle labels, which provide intensely fluorescent detection reagents.

The original polymeric latex particles still are widely used for separation and detection. Polymers provide a matrix that can be swollen for embedding other molecules in their core, such as organic dyes or fluorescent molecules. Even nanoparticle quantum dots can be incorporated into larger latex particles to form highly fluorescent composite microparticles.

Such fluorescent or dyed latex particles are useful in multiplexed detection systems using suspension arrays (e.g., Luminex technology; Armstrong *et al.*, 2000). In this application, dyes are incorporated within the beads having a range of different spectral properties to create a series of different colored particles. In addition, changing the amount of dye molecules within the beads or blending two or more dyes in a single particle population can form a gradient of different color compositions. Particle subpopulations of a particular color or emission wavelength and intensity then can be used to identify the type of target being measured in a suspension assay. Mixing together in a single solution such particle subpopulations having different colors permits multiple targets to be assayed simultaneously, wherein each particle type is identified by its color and correlated to the analyte being targeted. Flow-cytometry-based instruments then are used to detect and measure the particle color and assay result.

Dyed particles also are commonly used in diagnostic lateral flow tests (like the common home pregnancy test), as the colors can be seen with the eye without the need for special detectors. In this type of assay, antibodies or antigens are coupled to the dyed particles and a sample solution applied to the test strip carries them along within a membrane. The particles then are captured at points in the membrane that represent either a control or a positive sample result. Large numbers of color particles docking at these points within the membrane create the visual lines associated with these disposable tests.

Polymeric particles can be constructed from a number of different monomers or copolymer combinations. Some of the more common ones include polystyrene (traditional "latex" particles), poly(styrene/divinylbenzene) copolymers, poly(styrene/acrylate) copolymers, polymethylmethacrylate (PMMA), poly(hydroxyethyl methacrylate) (pHEMA), poly(vinyltoluene), poly(styrene/butadiene) copolymers, and poly(styrene/vinyltoluene) copolymers. In addition, by mixing into the polymerization reaction combinations of functional monomers, one can create reactive or functional groups on the particle surface for subsequent coupling to affinity ligands. One example of this is a poly(styrene/acrylate) copolymer particle, which creates carboxylate groups within the polymer structure, the number of which is dependent on the ratio of monomers used in the polymerization process.

Many of the common polymer particle types present a surface with hydrophobic character, such as the polyaromatic styrene-containing ones. These in particular are better used with modifications on the particle surface to add charge or hydrophilicity, which masks the underlying polymer core. Some constructs of this type provide a grafted hydrophilic surface to limit the degree of nonspecific binding and form a more biocompatible particle, which won't denature or bind protein through unwanted hydrophobic interactions. Some polymer particles are made from hydrophilic monomers and display better biocompatibility. HEMA is very hydrophilic due to an abundance of hydroxyl groups, and its properties are extremely biocompatible without further surface derivatization.

Inorganic particles are used extensively in various bioapplications, too. Gold nanoparticles long have been used as detection labels for immunohistochemical (IHC) staining and lateral flow diagnostic testing. These dark, dense particles provide single particle detection capability

for sensitive staining techniques, especially in microscopy applications. In addition, the ease at which clumps of gold particles can be seen visually makes their use in diagnostic strip tests a very popular alternative to dyed latex particles (see Chapter 24 for a thorough review of gold conjugation methods).

Invariably, the use of particles in bioapplications involves the attachment of affinity capture ligands to their surface, either by passive adsorption or covalent coupling. The coupling of an affinity ligand to such particles creates the ability to bind selectively biological targets in complex sample mixtures. The affinity particle complexes thus can be used to separate and isolate proteins or other biomolecules or to detect specifically the presence of these targets in cells, tissue sections, lysates, or other complex biological samples.

The reactions used for coupling affinity ligands to nanoparticles or microparticles basically are the same as those used for bioconjugation of molecules or for immobilization of ligands onto surfaces or chromatography supports. However, with particles, size can be a major factor in how a reaction is performed and in its resultant reaction kinetics. Since particle types can vary from the low nanometer diameter to the micron size, there are dramatic differences in how such particles behave in solution and how the density of reactive groups or functional groups affects reactions.

2. Particle Characteristics and Stability

Particle size, surface composition, and density directly affect how a particle behaves in suspension. This in turn affects coupling protocols, especially in the handling and washing techniques used for particles during the conjugation process. Larger particles of micron size generally will settle over time just in normal gravity. As particle size decreases, however, a point is reached that a true colloidal suspension may occur, wherein the particles won't separate no matter how long they sit in suspension. This typically happens when particle size gets to about 100 nm, and Brownian motion causes water molecules to collide with particles with high enough force-to-mass ratios to prevent them from settling under gravity. Many dense particles of less than 100 nm, such as silica, still can be separated from solution using a bench-top centrifuge, except as particles approach the size of biological macromolecules, or around 10 nm, at which point an ultracentrifuge would be required for separation. For a comparison of particle sizes and how they contrast to biomolecules (see Figures 14.1 and 14.2).

The forces acting on particles in suspension mainly can be explained by the Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory, which describes the attractive van der Waals forces and repulsive electrostatic forces affecting their stability (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). A corollary to this theory indicates that for hydrophobic surfaces, adding carboxylates can result in a stabilization of particles in solution and a prevention of aggregation through charge repulsion. Of course, this is true provided the pH of the solution is maintained above about pH 5, so that the carboxylates are not protonated, and their negative charge character is maintained. Often, without the presence of surface charge to create significant repulsive effects, many particles of commercial interest would aggregate and fall out of suspension rather quickly due to hydrophobic surface interactions and van der Waals forces (electrostatic attractions). The smaller the particle, the more significant the van der Waals attractive forces may become.

By contrast, relatively hydrophilic particles like those made of pHEMA may maintain colloidal stability even at small size due to the "repulsive" effects of a water of hydration layer,

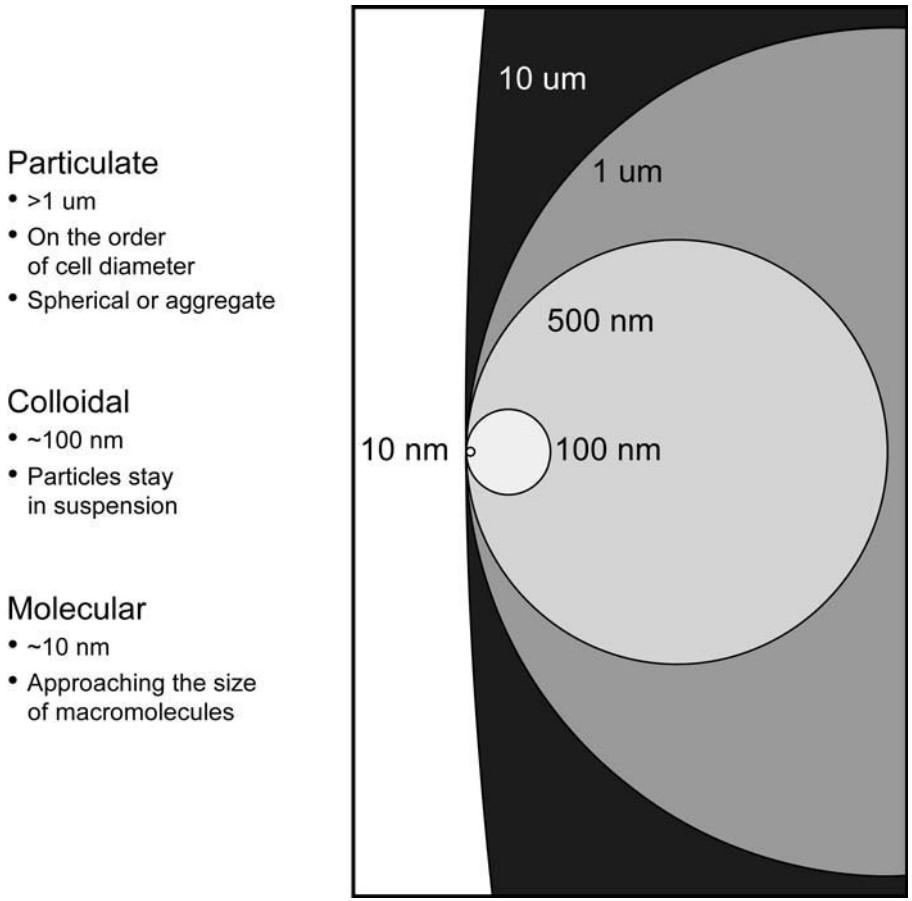


Figure 14.1 Particles commonly used in biological applications can range in size over three orders of magnitude, from as small as macromolecules (~10 nm) to approximately the diameter of cells (10 μm). The diameter of a particle population dramatically can affect its behavior in solution.

which forms around each particle in aqueous solution and is energetically difficult to remove or penetrate (Figure 14.3). A closely bound water layer apparently is the result of hydrogen bonding and dipole interactions with polar surface groups, such as the hydroxyls on pHEMA particles. Many such hydrophilic particles can display stability at high salt concentrations, and even if DLVO theory may predict aggregation (Molina-Bolívar *et al.*, 1998).

The degree and type of surface charge can dramatically affect particle behavior in suspension. Latex particles that have a very low density of charged groups, for instance, may still present hydrophobic polymer surface areas large enough to cause instability. The type of charged groups on the particle surface directly can affect the magnitude of charge repulsion. In particular, the activation of carboxylate particles using an EDC/sulfo-NHS two-step reaction (Chapter 3, Section 1.2) will temporarily replace the negatively charged carboxylates with negatively charged sulfonates. The sulfonate groups on the sulfo-NHS ester intermediates create a stronger negative charge on the particle surface than the original carboxylates. In some cases, the increase in negative charge

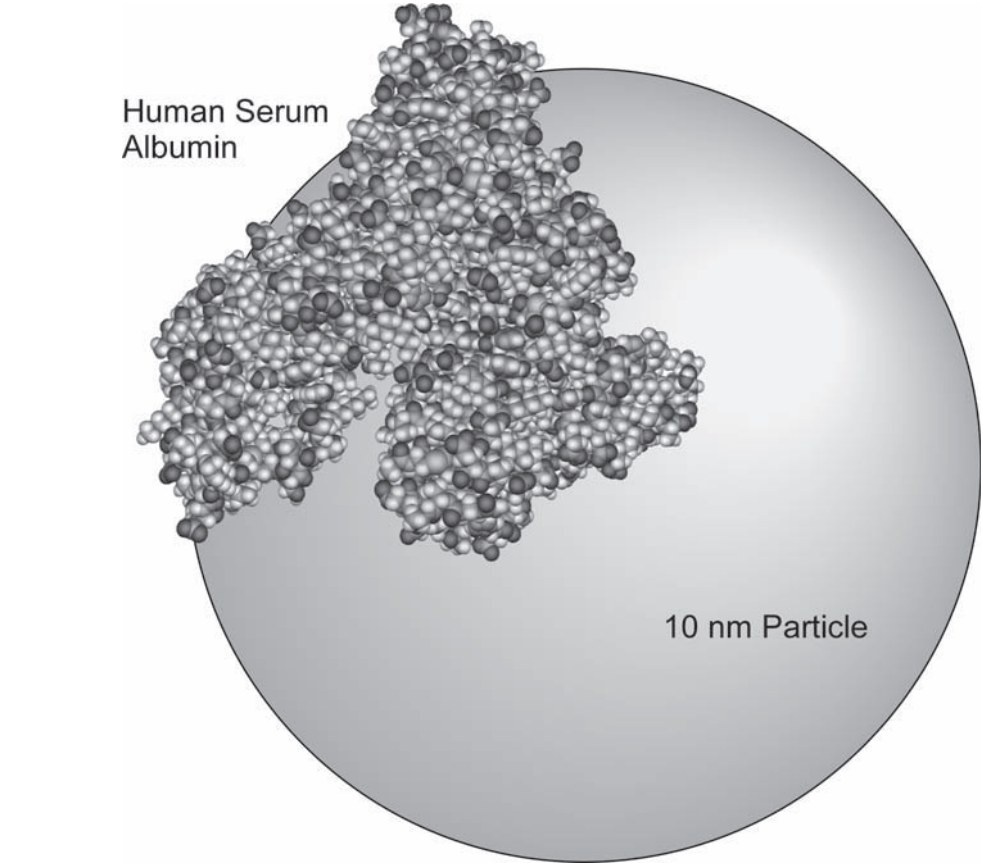


Figure 14.2 Comparison of the relative size of a 10 nm particle and the diameter of a typical protein, human serum albumin, at about 7 nm.

repulsion can result in an inability to pellet the particles by centrifugation after the activation step, even if the particles could be separated by centrifugation before activation. This was true for 40 nm carboxylated silica nanoparticles in our hands (unpublished observations).

The charge repulsion effects between particles can be severely affected by the buffer and salt composition of the solution they are suspended in. Charges can be eliminated or neutralized by ionizable groups being protonated or unprotonated or by the concentration of ions in solution. For instance, lowering the pH of an aqueous solution below the pK_a of the surface carboxylates will result in them being protonated. With most particles, especially hydrophobic ones, this will cause particle aggregation due to loss of surface negative charge. Similarly, a high salt concentration can effectively mask the charge character of a carboxylated particle by having too many positively charged ions associated with the surface charges. Most particle types that are stable in suspension due to like charge repulsion can be made to aggregate if the pH is changed or the buffer or salt concentration is too high. Part of the challenge of successfully working with

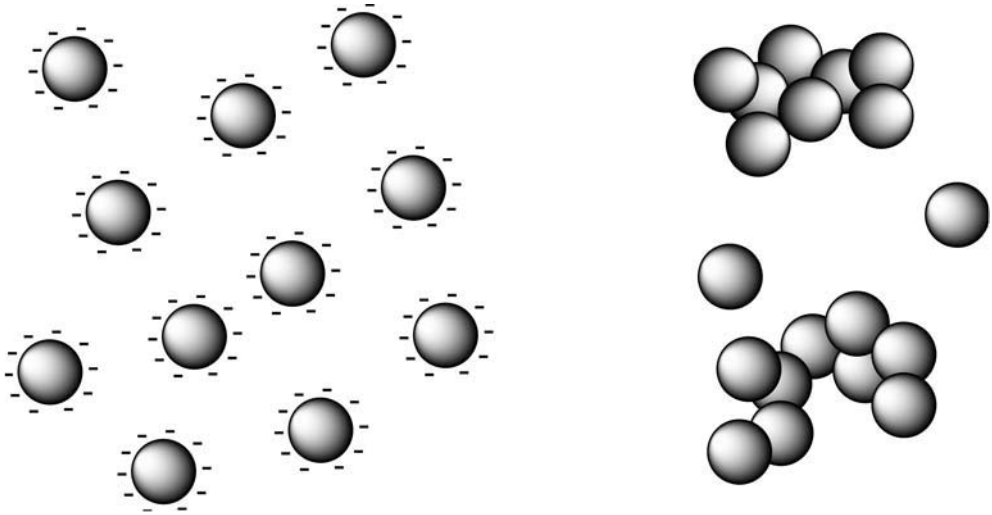


Figure 14.3 Small particles often are stabilized in aqueous solution by like charge repulsion, which prevents particle aggregation and precipitation.

small particles is to maintain optimal solution characteristics to keep the particles dispersed throughout the conjugation process. This includes all activation, coupling, and washing steps that are used to conjugate an affinity ligand and subsequently use it in its intended application. Therefore, for each particle type being used to conjugate a ligand, one should first become knowledgeable of its physical and solution characteristics before doing any coupling reactions.

Another aspect of particle handling that should be worked out before actually coupling an affinity ligand is the best procedure to wash the particles and remove unreacted ligands or by-products of a coupling reaction. With small quantities of particles, most often, this can be done by centrifugation or if working with very small nanoparticles, by gel filtration or size exclusion chromatography. Membrane filtration also is another option, especially for larger micron-sized particles. If the solution volume and quantity of particles is large enough, then tangential flow filtration is a viable alternative, too.

Although many particle types in theory can be separated from solution by centrifugation, some particles may become irreversibly aggregated upon pelletting and should not be centrifuged under any circumstances. The relative hydrophobicity and degree of charge on a particle's surface directly affects their tendency to aggregate, and this property to a large extent governs whether the particle population can be resuspended successfully after centrifugation. In particular, particles that contain higher densities of like surface charge, which functions to repulse and keep individual particles away from one another, typically can be resuspended successfully after centrifugation. To aid in re-suspension, centrifuged particles should be subjected to sonication, either by using a bath sonicator or a sonic probe to disrupt the pellet. Vortex mixing usually should be avoided, as it is not very effective at re-dispersing small particles pelletted by centrifugation. Before using centrifugation or any other separation technique, it is best to contact the manufacturer to see if they offer any guidelines for handling the particles.

3. Particle Concentration

If individual particles in suspension are considered the equivalent of discrete molecules, then the molar concentration of a given particle suspension can be calculated based on the known particle diameter, density, and the mass of particles present. This allows particles to be treated similarly to other biomolecules with respect to determining concentration for conjugation purposes. However, there are important differences that should be realized when working with particles as opposed to working with soluble macromolecules, like proteins. Since common commercial particles can vary in size from the molecular range (approximating the size of an antibody or ~10 nm diameter) to a scale 1,000 times larger (or approaching the size of a cell at 10 μm), a change in diameter affects the concentration of particles as well as the effective concentration of surface functional groups present in suspension.

In general, as particle size decreases, the molar concentration of particles in a constant volume of solution increases (for a given mass of particles). For instance, a 1 mg quantity of 1 μm latex microspheres represents far fewer particles than a 1 mg amount of 50 nm nanoparticles. Thus, the effective molar concentration of nanoparticles in solution will be much greater than the concentration of the same mass of microparticles (if both are suspended in the same volume of solution). In addition, as particle size decreases, the ratio of a particle's surface area to mass increases. This means that the total surface area available for conjugation on the nanoparticles is much greater than the total surface area present on the microparticles. If both particles contain the same functional groups on their surfaces for coupling affinity ligands (i.e., carboxylates), then the effective concentration of these groups in solution for the nanoparticles is much greater than the concentration of the same groups in a given solution for the microparticles (assuming both have about the same surface density or "parking area" of the carboxylate functional groups).

Thus, most conjugation reactions done with nanoparticles should take into account a potentially greater reactivity than the same reactions done using microparticles, due to the higher effective concentration of functional groups present in solution for the nanoparticles. As particle size decreases and particle concentrations increase, the available surface area increases, and the effective concentration of reactive groups increases along with it. All of these factors must be considered when optimizing conjugation reactions to particles. This means that an optimal protocol for coupling proteins to 1 μm carboxylated microspheres most likely will have to be re-optimized for use with carboxylated nanoparticles.

The following sections discuss many of the major particle types and provide bioconjugation options for the coupling of ligands to the surface of functionalized particles. Some additional nanoparticle constructs, including gold particles, dendrimers, carbon nanotubes, Buckyballs and fullerenes, and quantum dots are discussed more fully elsewhere (see Chapter 7; Chapter 9, Section 10; Chapter 15; and Chapter 24).

4. Polymeric Microspheres and Nanospheres

Perhaps the most common particle type used for bioapplications is the polymeric microsphere or nanosphere, which consists basically of a spherical, nonporous, "hard" particle made up of long, entwined linear or crosslinked polymers. Creation of these particles typically involves an emulsion polymerization process that uses vinyl monomers, sometimes in the presence of

divinyl crosslinking monomers. Larger microparticles usually are built from successive polymerization steps through growth of much smaller nanoparticle seeds. The investigation of particle surfaces by high-resolution electron microscopy often can reveal the presence of numerous entangled, amorphous nanoparticles making up the morphology of much larger nanospheres or microspheres. Instead of being smooth spheres, the surface of most polymeric particles actually is torturous and made up of many craters, pits, and crevasses, which may appear featureless under lower resolution imaging.

Some of the most common forms of polymeric particles consist of polystyrene or copolymers of styrene, like styrene/divinylbenzene, styrene/butadiene, styrene/acrylate, or styrene/vinyltoluene. Other common polymer supports include PMMA, polyvinyltoluene, and pHEMA and the copolymer, poly(ethylene glycol dimethacrylate/2-hydroxyethylmetacrylate) [poly(EGDMA/HEMA)] (Ayhan *et al.*, 2002) (Figure 14.4). The types of monomers used to form the polymers ultimately govern the final particle characteristics. If the monomers are hydrophobic, such as those that form most of the styrene-based particles, then the resultant particle surfaces will be hydrophobic, as well. The inclusion of hydrophilic monomers, including charged groups and polar constituents, will create more hydrophilic character on the surface. In addition, since many particles are polymerized in the presence of detergents, particles typically have detergent molecules non-covalently adsorbed on their surfaces, unless they have been purposely removed by extensive cleaning after polymerization.

Polymeric particles traditionally have been called "latex" beads or spheres, probably from the classic definition of an "emulsion of rubber or plastic globules in water". However, due to

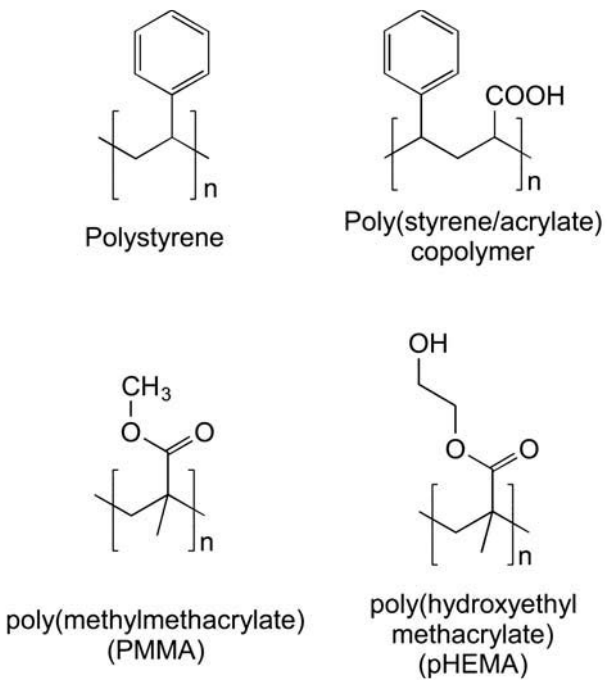


Figure 14.4 Some of the most common particles consist of these polymers.

the polymeric diversity of these particles, even of those that consist of a styrene base, it is best to identify the particle type by its exact chemical composition.

Polymeric particles also can be further diversified by their surface compositions. Even traditional polystyrene particles contain additional groups on their surfaces due to the polymerization reaction used to create them. For instance, nearly all such particles contain some negatively charged sulfonate groups, which are the result of the catalyst, persulfate used to initiate the polymerization process. In addition, many particles contain non-covalently adsorbed detergent molecules that can add amphipathic character to the particle surface, depending on the type of detergent used during the polymerization reaction. Detergents can be removed by extensive washing after the manufacturing process, but this may be a source of variability from manufacturer to manufacturer.

Another way of altering surface characteristics is through the purposeful addition of secondary polymers onto a polymeric core by graft copolymerization, adsorptive coating, or through covalent attachment of another polymer type. Adding hydrophilic coatings onto hydrophobic particle cores often is done to promote biocompatibility and limit nonspecific interactions with proteins or other biomolecules. Examples of hydrophilic coat polymers that have been used for this purpose include poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(acrylic acid), poly(methacrylic acid), poly(acrylamide), and poly(vinyl pyrrolidone). Hydrophilic modification of hydrophobic particle cores can aid in blocking the nonspecific binding potential that the core may have toward proteins or other biological molecules. It also can alleviate the tendency of particles to aggregate in aqueous solution by creating a hydrated shell around each particle, which helps keep them separated and in suspension. By example, Harper *et al.* (1995) describe the copolymerization of a poly(ethylene oxide) methacrylate monomer (PEG-type) to create polystyrene particles with hydrophilic surfaces for the attachment of cells.

Covalent attachment of hydrophilic spacer arms to particles is another way of masking the hydrophobic character of the underlying surface while making a particle more biocompatible. Spacers of this type are typically short organic molecules containing a number of polar groups along the chain, which are able to interact with the surrounding aqueous environment and limit nonspecific protein adsorption on the particles. A common spacer arm construction consists of a PEG chain that also may include a short hydrophobic straight chain linker, which attaches to the particle surface. To provide an attachment site for affinity ligands to the spacer, a functional group can be included on the outer end of the PEG polymer, such as a carboxylate group. These functional groups provide sites for covalent coupling of affinity ligands, including proteins, while the PEG chain creates a hydrophilic "lawn" to limit nonspecific interactions. The best configuration of this spacer type is to have about 10 percent of the spacers terminate in a carboxylate group while the rest of them terminate in a PEG hydroxyl or a PEG methyl ether group. This design has been used with success with other surface types, such as gold nanoparticles or planar surfaces (Prime and Whitesides, 1991), and it can be adapted to polymer particles with little difficulty (Figure 14.5).

4.1. Passive Adsorption

One of the simplest methods of attaching biomolecules to hydrophobic polymeric particles is the use of passive adsorption. Some of the earliest examples related to the use of particles in immunoassays include the use of non-covalently adsorbed antibody or antigen onto latex microspheres. Protein adsorption onto hydrophobic particles takes place through strong interactions

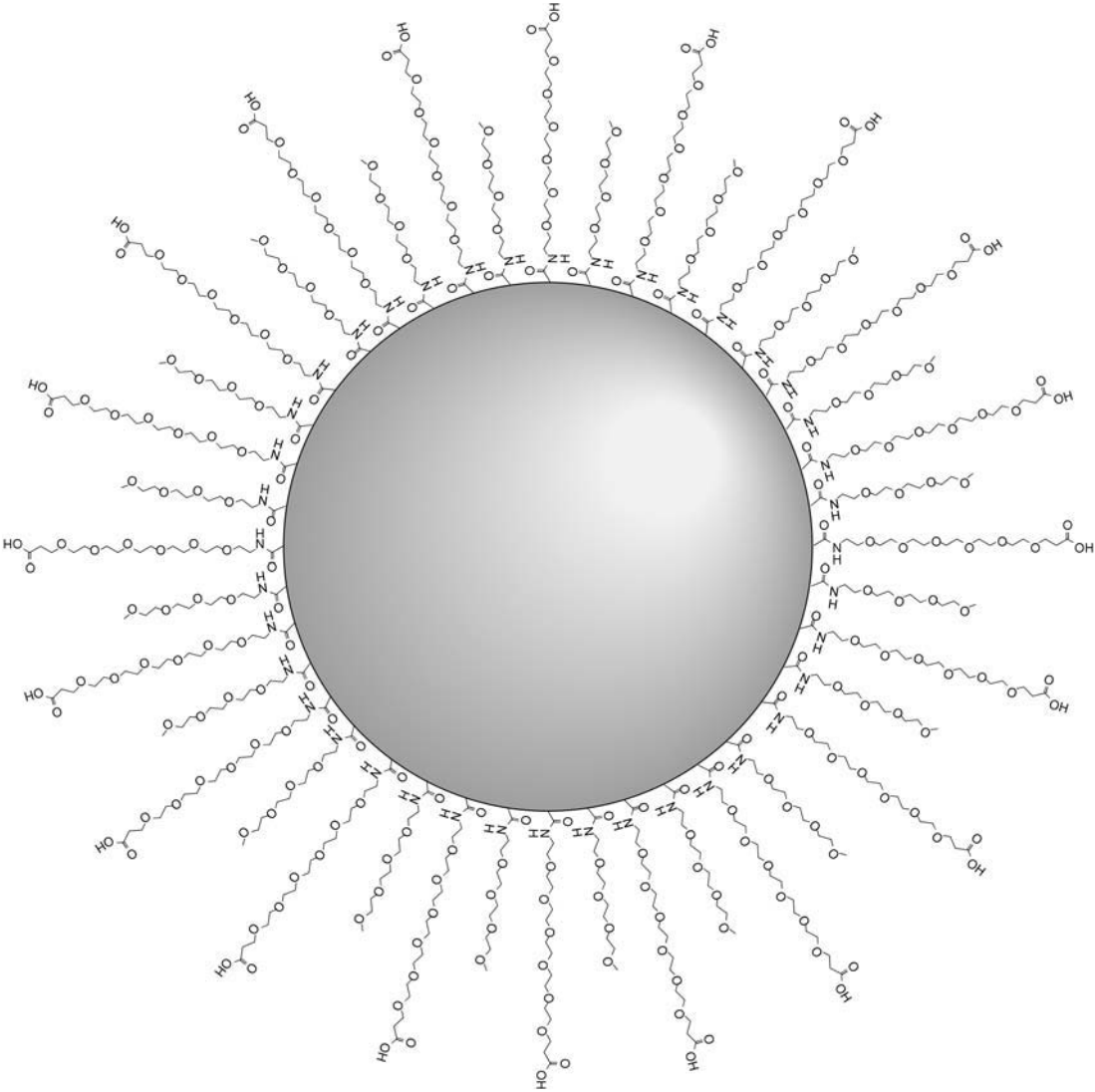


Figure 14.5 A method of making particles biocompatible includes the use of PEG-based spacers. A lawn of mPEG molecules in interspersed with some longer PEG chains that terminate in carboxylate groups for coupling amine-containing molecules. The result is an extremely hydrophilic surface with low nonspecific binding.

of non-polar or aromatic amino acid residues with the surface polymer chains on the particles with concomitant exclusion of water molecules. Since proteins usually contain hydrophobic core structures with predominately hydrophilic surfaces, their interaction with hydrophobic particles must involve significant conformational changes to create large-scale hydrophobic contacts. These conformational changes often result in complete denaturation of the first protein layer to be adsorbed onto the particles. Subsequent protein molecules which bind and add to this initial layer are adsorbed through protein–protein binding or aggregation events, which ultimately

result in the formation of protein clusters in which the outer layers of protein have more of a native conformation and activity (Butler, 2000a, b).

Due to this tendency of proteins to form multilayer coatings on hydrophobic surfaces, the amount of excess protein beyond the particle saturation point (theoretical monolayer density for the protein type) added during adsorption processes should be limited. If extremely large excesses of protein are added to particles, highly unstable constructs may result which may continually leach off loosely adsorbed protein. Most protocols recommend a protein concentration of about 3–10 × excess of protein over the calculated monolayer concentration for the particles used. An estimate of the maximal amount of a given protein that can bind as a monolayer to a particle surface can be determined by the fact that bovine serum albumin (BSA) (MW 67 KDa) has an adsorption capacity of about 3 mg/m^2 of particle surface. A larger protein like an antibody (IgG; MW 150 KDa) has a maximal adsorption density of about 2.5 mg/m^2 of particle surface. To translate this into a real-world example, 1 g of 1 μm polystyrene microspheres can adsorb about 18 mg BSA or about 15 mg IgG (Cantarero *et al.*, 1980). Knowledge of a particle's surface area per gram will permit reasonable estimates of maximal adsorption capacity for a given protein relative to the size of an antibody or albumin molecule.

Passive adsorption has been studied extensively as it relates to the immobilization of antibody molecules onto microplates (polystyrene) or microspheres (hydrophobic beads of various compositions). Although the denaturing effect of passive adsorption of proteins onto surfaces was known since 1956 when Bull studied the adsorption of albumin onto glass, it was not widely recognized as being detrimental until a number of more recent studies were published. See in particular Butler *et al.* (1992) which investigates the physical and functional behavior of capture antibodies adsorbed on polystyrene, Butler (2000a) which provides a summary of the effects of the adsorption of various proteins on hydrophobic surfaces, Cantarero *et al.* (1980) which discusses the adsorptive characteristics of proteins for polystyrene surfaces, and Butler *et al.* (1997) which compares the effect of passive adsorption on the antigen specificity of immobilized antibody molecules.

The detrimental effects on biomolecules as a result of passive adsorption onto hydrophobic surfaces can be dramatic. Butler *et al.* (1993) determined that over 90 percent of monoclonal antibodies and about 75 percent of polyclonal antibodies against fluorescein were denatured upon adsorption onto polystyrene surfaces. In addition, Bagchi and Birnbuam (1981) observed that IgG was optimally adsorbed to poly(vinyltoluene) particles at a pH value near the antibody's pI, indicating that the interactions were entirely hydrophobic in nature and didn't depend on charge interactions. Once the antibody was adsorbed, however, it was tightly bound to the surface, provided that the pH was maintained at the initial adsorption point. If any pH cycling was done, such as may occur with some wash steps, the adsorbed antibody had a tendency to leach off the surface, demonstrating the non-covalent nature of the interaction.

Protein adsorption to hydrophobic polymer particles thus should be done at or near the isoelectric point of the particular protein to assure the highest density of protein is attached to the particles. Proteins at their isoelectric point exist in the most collapsed conformation possible, because charge repulsion effects don't play as significant a role on overall globular structure. Upon adsorption under isoelectric conditions, each protein then can bind to the polymer surface at maximal density. Therefore, most suggested buffer cocktails used for passive adsorption recommend pH conditions somewhere in the pI range of the protein. Since many proteins have a range of different biological modifications (post-translational) resulting in a range of pI values, some optimization may need to be done to determine the best pH conditions to use for an adsorption process.

Another factor to consider is the amount of protein available for adsorption. If expensive antibody in very small amounts is used to passively adsorb to polymeric particles, often there is not enough antibody available to saturate the particle surface. In this case, another carrier or blocking protein may be added to the mixture to take up otherwise unoccupied sites on the particle surface. This will prevent particle aggregation that may occur if low concentrations of protein are used in the adsorption process. Carrier proteins frequently used for this purpose include albumin or polyclonal IgG pools, such as bovine gamma globulin. Other standard protein blocking agents also may be used for blending with the desired antibody, such as casein, non-fat dried milk proteins, fish serum, etc.

The polymeric particle composition also plays a significant role in the amount of protein adsorbed and its stability and activity after immobilization. In general, copolymer blends in which a hydrophobic monomer is polymerized with a charged monomer (such as acrylic acid or methacrylate) create a surface that has both hydrophobic character along with areas of charge. Proteins adsorbed onto these copolymer particles interact with the surface through hydrophobic interactions and positive–negative charge attraction. The result is less protein denaturation upon adsorption and better retention of activity after immobilization. A comparison of protein adsorption onto pure polystyrene and several other copolymer particle types by Bale *et al.* (1989) resulted in the following order of binding affinity:

Polystyrene > polystyrene/PMMA copolymer > PMMA > polystyrene/polyacrylic acid copolymer

Thus, pure hydrophobic polymer compositions adsorb proteins very strongly, but have the greatest tendency to denature protein at the initial protein–surface interface. Copolymers containing some polarity or negative charge character bind protein less avidly, but result in better retention of activity. The inclusion of some polar hydrophilic groups within an overall hydrophobic surface structure has been used with success for polystyrene microplates, as most of the so-called high binding plates contain a population of polar constituents on the well surfaces, allowing both hydrophobic and charge or dipole interactions to occur.

The following protocol for passive adsorption is based on methods reported for use with hydrophobic polymeric particles, such as polystyrene latex beads or copolymers of the same. Other polymer particle types also may be used in this process, provided they have the necessary hydrophobic character to promote adsorption. For particular proteins, conditions may need to be optimized to take into consideration maximal protein stability and activity after adsorption. Some proteins may undergo extensive denaturation after immobilization onto hydrophobic surfaces; therefore, covalent methods of coupling onto more hydrophilic particle surfaces may be a better choice for maintaining native protein structure and long-term stability.

Protocol

1. Dilute the particles to a mass concentration of 10 mg/ml (1 percent) in coating buffer at a pH near the pI of the protein being adsorbed. If particle aggregation becomes a problem, the initial particle concentration may be reduced to 5 mg/ml. Some typical coating buffer suggestions include: (a) 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS); (b) 50 mM sodium borate, pH 8.5; (c) 50 mM sodium acetate, pH 3.6–5.6; (d) 25 mM MES [2-(*N*-morpholino)ethane sulfonic acid], pH 6.1; or (e) 50 mM sodium bicarbonate,

pH 8.5–9.5. NaCl may be added to any of these buffers at a final concentration of 0.15 M to promote protein stability, if required. However, avoid the use of detergents or chaotropic agents, as these will compete with protein adsorption or reduce the strength of hydrophobic interactions, thus limiting the yield.

2. Dissolve the protein to be adsorbed in coating buffer at a concentration such that when the solution is mixed with the particle suspension, the appropriate protein concentration is reached to obtain an excess of about 3–10 × over the maximal monolayer density for that protein on the particles (see previous discussion). Note: A good strategy is to perform a protein titration study, wherein a range of protein concentrations are tried with a given particle quantity. For instance, using a 300 nm latex particle, a suitable set of trial experiments would employ protein concentrations in the range of about 10–200 μg protein/mg particles. Plotting the amount of protein charged to the coating reaction versus the amount bound will provide data to identify the optimal protein concentration to be used.
3. With rapid mixing, add protein solution to the particle suspension. For small volumes, mixing can be done by pulling the solution up and down in a pipette tip or by vortexing. For larger volumes, a stir bar or paddle may be used. Some protocols recommend adding the particles to the protein solution to obtain the best initial mixing rate, thus assuring even particle coating throughout the particle suspension. The key is rapid and efficient mixing to create a homogeneous mixture of protein and particles, so adsorption can occur uniformly on each particle.
4. Mix the suspension for 1 hour at room temperature.
5. Remove excess protein by centrifugation or tangential flow filtration. Many particles may be pelletted successfully using a tabletop centrifuge, especially if the particle size is above about 150 nm in diameter. If particle clumping is a problem upon pelletting or the particle size is too small to be effectively pelletted, then filtration is the better alternative. Centrifuge and wash the particles at least twice with coating buffer to assure complete removal of non-bound protein. Complete re-suspension of the particles may be accomplished by the use of sonication, especially through the use of a sonic probe dipped into the solution.
6. Resuspend the particles to a final concentration of 1 percent in coating buffer containing a preservative. Avoid changing the composition of the storage buffer from that used to coat the protein, as any pH or compositional changes often result in elution of some of the adsorbed protein.
7. Determine the amount of adsorbed protein on the particles by using a suitable protein assay technique, such as the bifunctional chelating agents (BCA) Protein Assay (Thermo Fisher).

4.2. Covalent Coupling to Polymeric Particles

Many particle types contain functional groups that are built into the polymer backbone and displayed on their surface. The quantity of these groups can vary widely depending on the type and ratios of monomers used in the polymerization process or the degree of secondary surface modifications that have been done. Some common particle functionalities are shown in Figure 14.6. Many of these functionalized particles can be used to couple covalently biomolecules through the appropriate reaction conditions (Illum and Jones, 1985; Arshady, 1993). For each type of particle, manufacturers may offer several different densities of functional groups for different applications.

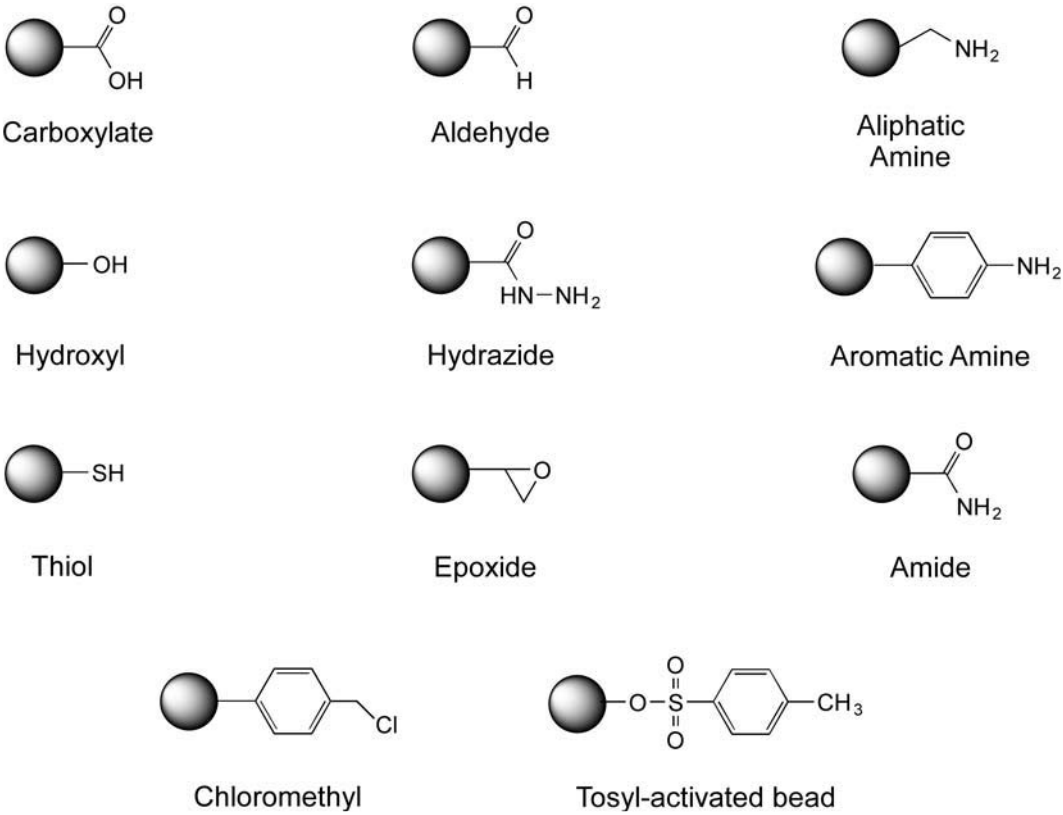


Figure 14.6 Common functional groups or reactive groups on particles that provide the ability to couple proteins or other ligands.

4.3. Coupling to Carboxylate Particles

One of the more common particle types is the carboxylate particle, which typically is created by copolymerization or grafting with acrylic acid or methacrylic acid monomers. However, the characteristics of carboxylate particles can vary between manufacturers and particle sources due to the differences in surface density of carboxylate groups present. A measurement of chemical density on particle surfaces is called the "parking area", and it refers to the average area in $Å^2$ occupied by each functional group. A brief survey of carboxylate particles from different manufacturers indicates that the average carboxylate parking area can vary widely from about 10 $Å^2$ (1 nm^2) to over 125 $Å^2$ (12.5 nm^2). One carboxylate group every 125 $Å^2$ is equivalent to about one carboxylate for every square having dimensions of 3.5 nm × 3.5 nm, or the space that might be occupied by one small protein on the particle surface (*note*: albumin (67 kDa) has an effective molecular diameter of about 7 nm, while an IgG molecule (150 kDa) has a diameter of about 11 nm). At a density of one carboxylate every 125 $Å^2$, there still may be considerable polymer surface exposed, which could be a source for nonspecific binding, especially if the underlying polymer structure has hydrophobic character. A protein coupled to

a carboxylate on such a particle could unfold through interactions with the exposed hydrophobic polymer surface and become denatured. Conversely, at the high end of carboxylate density, there would be about one carboxylate present for every 1 nm^2 of particle surface. For the covalent coupling of proteins, this high-density surface would provide more reactive sites for conjugation, while effectively masking the underlying polymer surface with negative charges. This helps to maintain particle suspension through negative charge repulsion and prevents nonspecific binding or protein denaturation.

Carboxylate particles can be activated by a number of strategies, which yield reactive intermediates capable of coupling with nucleophiles in proteins and other biomolecules. Figure 14.7 illustrates some of the reactions that can be used for coupling amine-containing molecules to carboxylate particles, all of which form stable amide linkages with the particle. The water-soluble reactions can be done in entirely aqueous conditions, both for activation and coupling. For some reactions, however, the activation process must be done under nonaqueous conditions to prevent hydrolysis of the activator or active intermediate. Activation in organic solvent is an option for particles that remain stable in nonaqueous conditions. Some polymer particles may unacceptably swell in organic solvent and they potentially could be damaged by such treatment.

By far the most common reaction strategy for coupling proteins and other amine-containing molecules to carboxylate particles is through an aqueous, carbodiimide-mediated process using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide), either in a single-step coupling reaction or in a two-step reaction that employs the addition of *N*-hydroxysuccinimide (NHS) or sulfo-NHS (see Chapter 3, Section 1.2 for additional information). Of all the crosslinking methods used in bioresearch applications today, this relatively simple coupling process is the most frequent conjugation reaction done with proteins. Using this reaction, carboxylate particles first are activated with the water-soluble carbodiimide EDC to create an intermediate ester. This ester is reactive directly with amines on proteins, but it also can be used with the addition of NHS or sulfo-NHS, which results in the formation of another intermediate, the NHS ester or sulfo-NHS ester. The formation of this second ester results in a more stable intermediate in aqueous solution than the one formed with EDC, therefore the secondary coupling reaction with proteins proceeds with higher yields than with the use of EDC alone (Figure 14.7). In addition, by forming the secondary (sulfo)NHS ester, excess EDC can be removed from the particles before adding protein, thus preventing carbodiimide-mediated protein polymerization due to the presence of both amines and carboxylates on most proteins (Staros, 1982; Borque *et al.*, 1994; Bonfield *et al.*, 2005).

For particle conjugation, it is important to maintain a repulsive force between particles to stabilize the colloidal suspension, even during the activation and coupling reactions. For this reason, the use of sulfo-NHS instead of NHS in this reaction results in an intermediate ester, which is strongly negatively charged. The sulfonates on the sulfo-NHS esters actually are more effective at keeping particles from aggregating than the original carboxylates; therefore, the reactive intermediate particles easily can be purified from excess reactants and then mixed with protein for coupling. If the non-sulfonated NHS is used in this reaction, the uncharged intermediate NHS ester may cause unacceptable particle aggregation, depending on the particle type, although uncharged NHS has been used widely with success.

The following protocols for coupling amine-containing molecules to carboxylated particles represent viable starting points for optimizing a method that works best for the particular molecule or protein being immobilized. The single-step method using EDC alone is appropriate for use in coupling molecules having one or more amines present without any carboxylates. It works

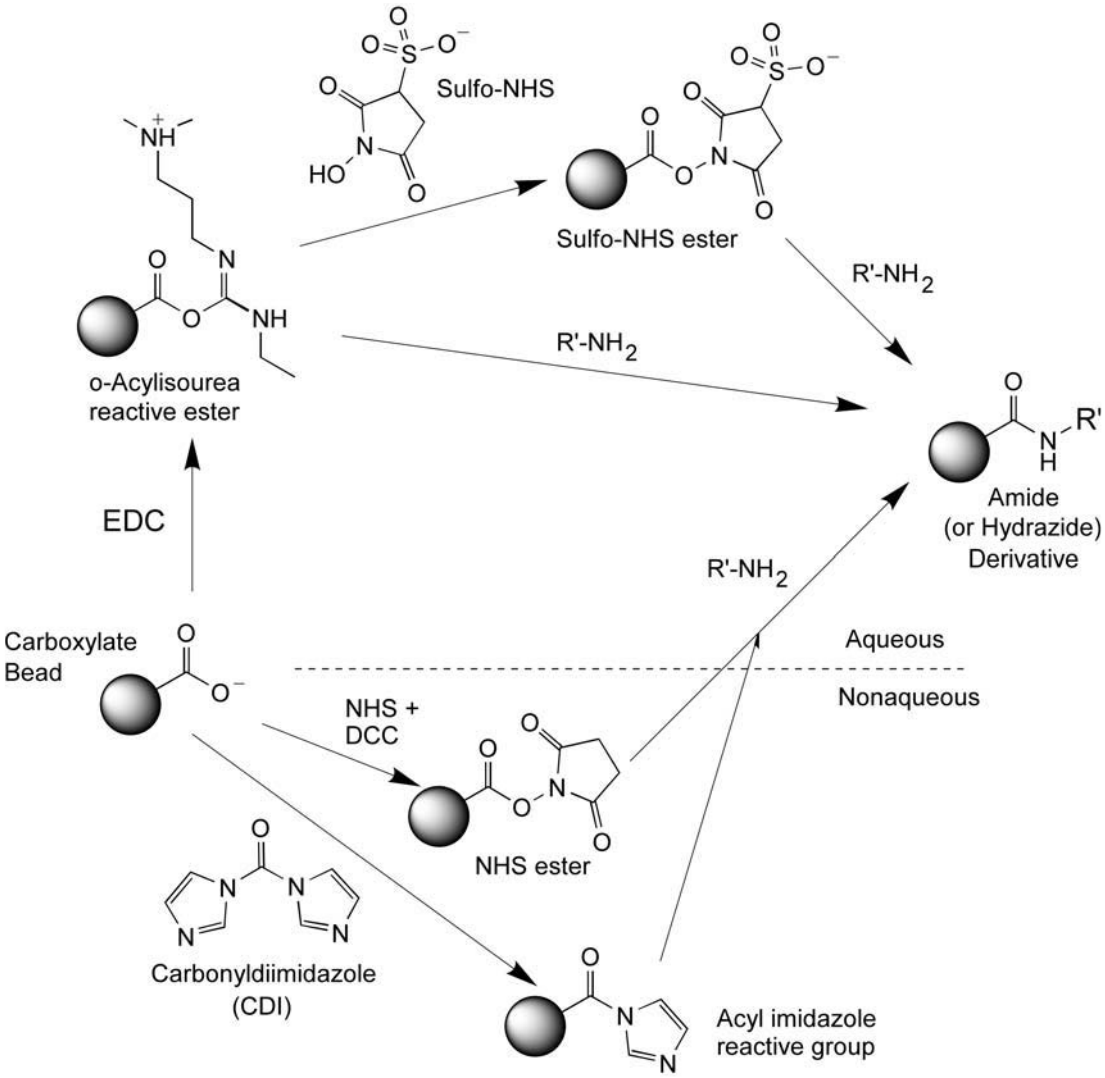


Figure 14.7 Carboxylate particles can be coupled to amine-containing molecules using a number of reaction strategies. The most frequently used method involves an aqueous two-step coupling process using EDC and NHS or sulfo-NHS to form an amide bond with a protein or other molecules. It proceeds through an intermediate (sulfo)NHS ester, which has better reactivity for coupling amines than the initial EDC-reactive ester. Organic solvent activation processes using NHS esters or acyl imidazole-reactive groups also can be used with solvent-stable particles to result in the same product with an amine-containing molecule.

especially well for small organic molecules, such as haptens, 5′-amino-modified oligonucleotides, and amine-containing steroid derivatives (Hager, 1974; Quash *et al.*, 1978; Nathan and Cohn, 1981; Fuller *et al.*, 2006). If the molecule being coupled has both amines and carboxylates, such as proteins, then it is best to use the two-step method, which eliminates excess EDC before the addition of ligand, otherwise unacceptable ligand polymerization may take place.

Single-Step EDC Coupling Protocol

1. Wash particles (e.g., 100 mg of 1 μm carboxylated latex beads) into coupling buffer (i.e., 50 mM MES, pH 6.0 or 50 mM sodium phosphate, pH 7.2; buffers with pH values from pH 4.5–7.5 may be used with success; however, as the pH increases the reaction rate will decrease). Suspend the particles in 5 ml coupling buffer. The addition of a dilute detergent solution may be done to increase particle stability (e.g., final concentration of 0.01 percent sodium dodecyl sulfate (SDS)). Avoid the addition of any components containing carboxylates or amines (such as acetate, glycine, Tris, imidazole, etc.). Also, avoid the presence of thiols (e.g., dithiothreitol (DTT), 2-mercaptoethanol, etc.), as these will react with EDC and effectively inactivate it.
2. Dissolve the amine-containing ligand to be coupled in 5 ml coupling buffer at a concentration sufficient to provide a 1- to 10-fold molar excess of ligand over the maximal calculated carboxylate group concentration for the amount and type of beads used. For particle manufacturers reporting a carboxylate concentration in meq/g, this is equivalent to μmol/mg.
3. Combine the ligand solution with the particle suspension and mix thoroughly.
4. Add 100 mg EDC and mix to dissolve. To facilitate faster dissolution, EDC may be dissolved immediately before use as a concentrated stock solution in reaction buffer and then an aliquot of this solution added to the particle suspension to obtain the correct final concentration.
5. React at room temperature 2–4 hours with mixing.
6. Wash the beads and resuspend them in coupling buffer containing 30–40 mM of an amine-containing hydrophilic quenching molecule to block excess reactive sites (i.e., ethanolamine or Tris).
7. Wash beads and store in an appropriate buffer containing a preservative.

Two-Step EDC/Sulfo-NHS Coupling Protocol

An alternative to this procedure was used by Kulin *et al.* (2002) for coupling antibodies to carboxylated microspheres, which provides different buffer conditions and activation with EDC without the use of sulfo-NHS or NHS.

Activation

1. Wash particles (e.g., 100 mg of 1 μm carboxylated latex beads) into coupling buffer (50 mM MES, pH 6.0). Suspend in 5 ml coupling buffer. The addition of a dilute detergent solution may be done to increase bead stability and prevent clumping (e.g., 0.01 percent SDS). Avoid the addition of any components containing carboxylates or amines (such as acetate, glycine, Tris, imidazole, etc.). Also, avoid the presence of thiols (e.g., DTT, 2-mercaptoethanol, etc.), as these will react with EDC and effectively inactivate it.
2. Add 100 mg of EDC and 100 mg of sulfo-NHS. Mix to dissolve. To facilitate faster dissolution, EDC and sulfo-NHS may be dissolved immediately before use as a concentrated stock solution in reaction buffer and then an aliquot of this solution added to the particle suspension to obtain the correct final concentration.
3. React for 15 minutes at room temperature.

4. Quickly wash beads 2 times with coupling buffer using centrifugation and resuspend using a sonic probe in 5 ml of the same buffer.

Coupling

5. Dissolve protein to be coupled in 5 ml coupling buffer at a concentration sufficient to provide 1- to 10-fold molar excess of ligand over the maximal calculated monolayer concentration for the amount and type of beads used. For particle manufacturers reporting a carboxylate concentration in meq/g, this is equivalent to μmol/mg. The optimal protein concentration should be optimized. *Note*: Too low a protein concentration may result in particle crosslinking. For coupling of expensive antibodies that may not be available in enough quantity to reach the optimal molar ratio on the particles, the addition of another protein (i.e., bovine gamma globulin or BSA) may be done to take up remaining reactive sites.
6. Combine the protein solution with particles and mix thoroughly.
7. React at room temperature 2–4 hours with mixing.
8. Wash beads with coupling buffer and resuspend in the same buffer containing 100 mM of an amine-containing hydrophilic quenching molecule to block excess reactive sites (i.e., ethanolamine or Tris).
9. Wash beads and resuspend in an appropriate buffer for storage.

4.4. Coupling to Amine Particles

Primary amine-containing polymeric particles are available from a number of manufacturers and have either aliphatic or aryl amine groups on their surface. Occasionally, a particle type may have secondary or tertiary amines present, but these should be avoided for covalent coupling, as primary amines typically give better reaction yields than secondary amines and tertiary amines are unreactive.

Primary amine particles may be used for covalent immobilization using a number of reaction routes (Figure 14.8). A carbodiimide-mediated coupling process may be used, as described above for carboxylate particles, but this time it is done by activation of a carboxylate group on the ligand and subsequent amide bond formation with the amines on the particles. A single step EDC reaction strategy will work well for small ligands containing one or more carboxylates with no amines. However, for carbodiimide-mediated protein coupling to amine particles, the protein first should be activated with EDC and sulfo-NHS according to the method of Grabarek and Gergely (1990) and then the activated intermediate added to the amine particles for conjugation (see Chapter 3, Section 1.2 for the activation protocol). This type of two-step reaction will prevent protein polymerization during the coupling process. Add a quantity of activated protein to the amine particles to result in a 1–10 × molar excess over the total molar quantity of amines present on all the particles used in the reaction.

4.5. Coupling to Amine Particles Using Crosslinking Agents

Another possible route to coupling ligands to amine particles is to use a bifunctional crosslinking agent to react with the amines and provide another reactive group at the other end to couple with the ligand. In this approach, virtually any reactive group desired can be formed on the

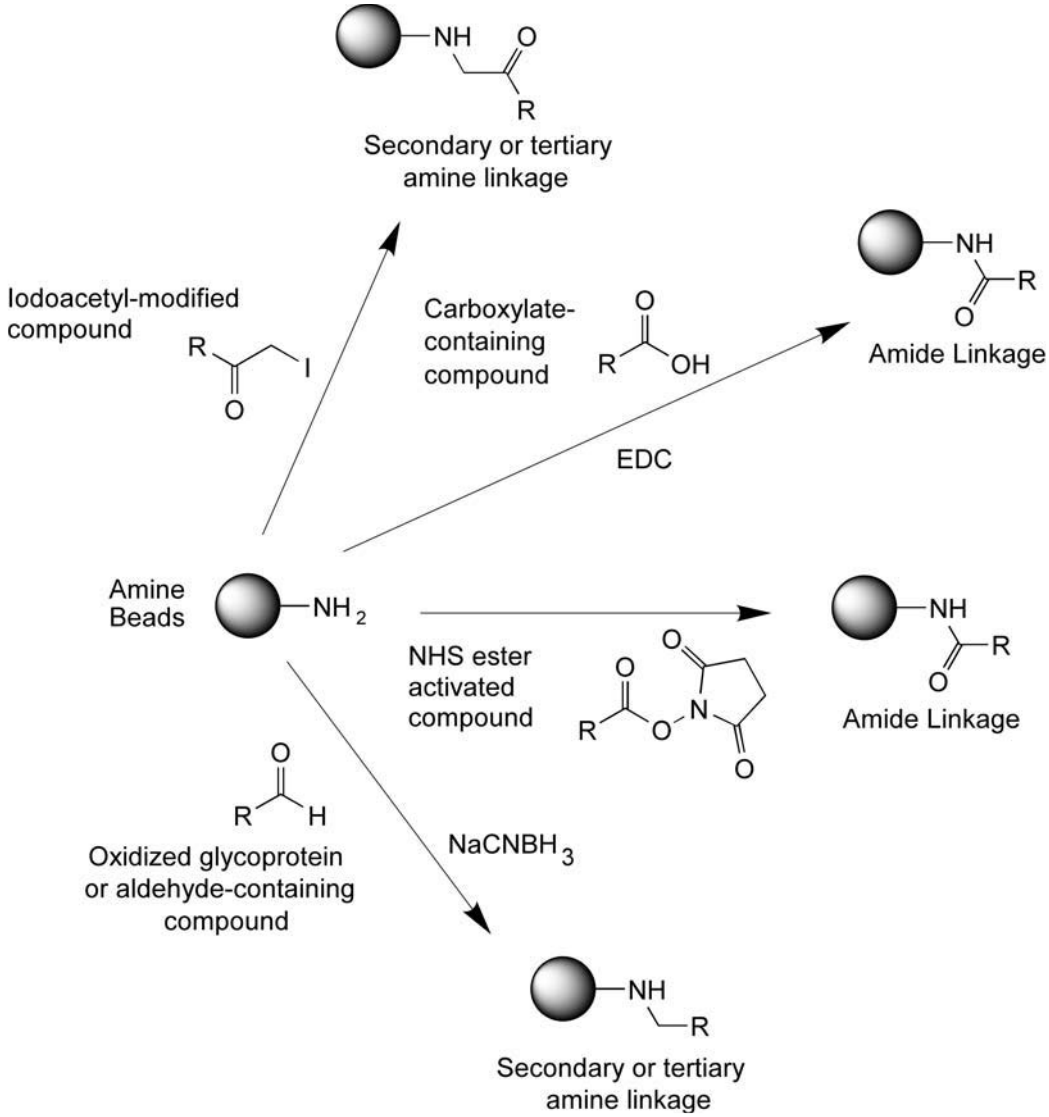


Figure 14.8 Amine-containing particles can be conjugated using alkylation or acylation reactions to result in secondary or tertiary amine linkages or amide bonds.

particles. Two strategies can be used with crosslinking agents: (1) use of a homobifunctional reagent, which contains the same reactive groups on either end or (2) use of a heterobifunctional compound, which contains different reactive groups on each end.

A homobifunctional amine-reactive compound can be used initially to modify the amine groups on particles, while leaving the remaining amine-reactive groups available to couple with ligands. This type of reaction must be done with the crosslinker in great excess to prevent polymerization of the amine particles themselves. There must be enough crosslinker present

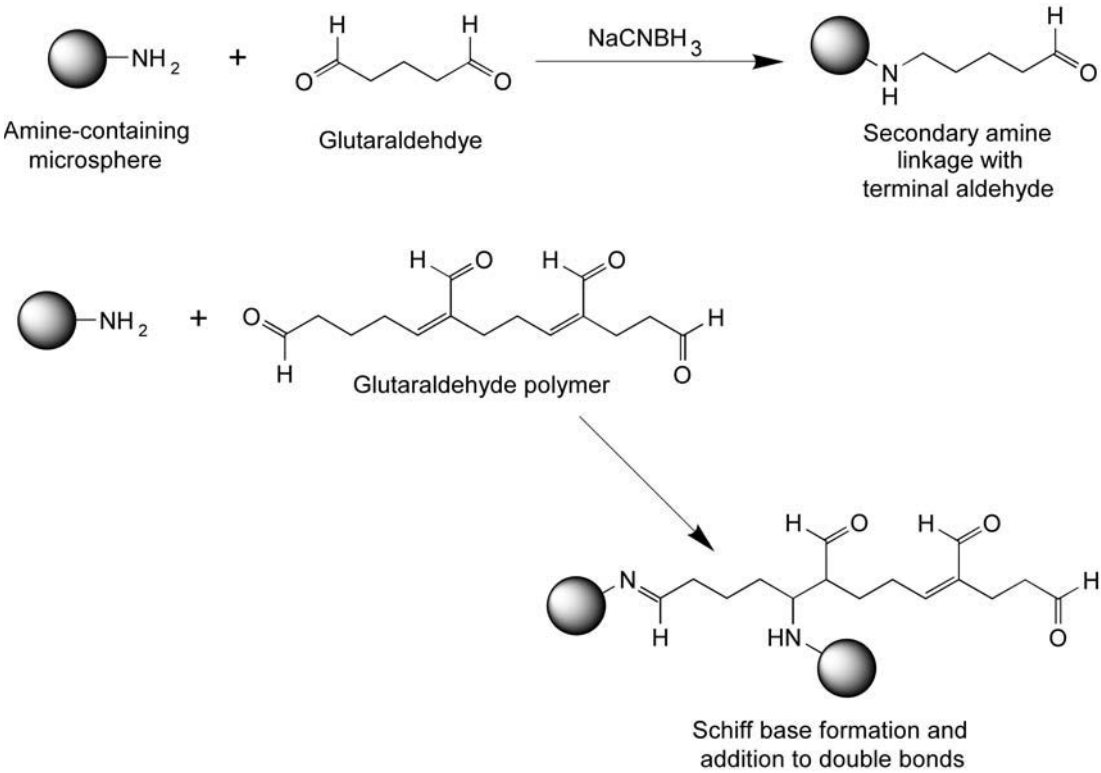


Figure 14.9 Glutaraldehyde conjugation to amine particles can proceed via two routes. Reaction of the aldehyde groups with the amines using sodium cyanoborohydride results in secondary (or tertiary) amine linkages with modifications containing a terminal aldehyde for further coupling to ligands. Alternatively, glutaraldehyde polymers can react with amine particles via addition to double bonds, resulting in a polymeric coating that contains both aldehydes and additional double bonds for further coupling with amine-containing molecules.

during the activation stage to avert the free end from attaching to another particle before that particle's amines are modified with other crosslinkers.

Once the amine particles are modified with a homobifunctional crosslinker, the excess reagent is removed so that when the ligand is added, it doesn't become polymerized, particularly if it has more than one amine (e.g., proteins). Homobifunctional crosslinkers containing NHS esters, imidoesters, or aldehyde groups on each end can be used for this type of coupling process. The following protocol illustrates this method using glutaraldehyde.

4.6. Glutaraldehyde

In one of the simplest methods, a homobifunctional amine-reactive crosslinker may be used to activate the surface for coupling with an amine-containing ligand (Figure 14.9). This has been done with success using glutaraldehyde or polyglutaraldehyde (Rembaum *et al.*, 1976; Rembaum *et al.*, 1978; Margel *et al.*, 1979; Kaplan *et al.*, 1983). The following protocol

describes a two-step coupling method using glutaraldehyde on amine particles based on the procedures of Kaplan *et al.* (1983) and Bang's Laboratories.

Protocol

1. Wash 10 mg of amine particles 3 times with 10 mM sodium phosphate, pH 7.4 (coupling buffer).
2. After the final wash, suspend the particles in coupling buffer containing 10 percent glutaraldehyde and mix well to dissolve.
3. React with mixing for 1 hour at room temperature.
4. Wash the particles with coupling buffer at least several times using centrifugation to remove excess glutaraldehyde. Resuspend in 1 ml of the same buffer.
5. Add the protein to be coupled to the particle suspension in an amount equal to 1–10 × molar excess over the calculated monolayer for the protein type to be coupled. Mix thoroughly to dissolve. Low concentrations of protein may result in particle aggregation, because a single protein molecule can react with more than one particle.
6. React with mixing for 2–4 hours.
7. Add to the particle suspension a final concentration of 0.2 M glycine (or another amine-containing quench molecule, such as ethanolamine or Tris) and 10 mM sodium cyanoborohydride. The blocking agent will couple to any remaining glutaraldehyde-reactive sites and the reducing agent will convert all the resultant Schiff bases into stable secondary amine linkages.
8. Remove excess protein and reactants by washing with coupling buffer at least 3 times using centrifugation. Store particles in a suitable buffer containing a preservative.

4.7. SPDP Coupling to Amine Particles

Another crosslinker-based method that has been used for coupling proteins to amine particles involves the use of *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP). This reagent contains an amine-reactive NHS ester and a thiol-reactive pyridyl disulfide group (Chapter 5, Section 1.1). Amine-containing particles can be activated by reaction with SPDP to form thiol-reactive derivatives. Thiol-containing proteins, such as partially disulfide-reduced antibodies, can be coupled to the activated particles in a two-step reaction. An alternative method was outlined by Illum and Jones (1985) that was based on Barbet *et al.* (1981) to couple SPDP modified amine particles to antibodies that were also modified with SPDP and then the pyridyl disulfide group reduced to form thiols. Mixing the thiolated antibody with the SPDP-activated particles results in covalent coupling via disulfide linkages (Figure 14.10).

Unlike the use of homobifunctional crosslinkers, heterobifunctional compounds usually don't have to be used in large excess with amine particles to prevent aggregation. This is due to the fact that only one of the ends of the crosslinker can react with the amines on the particles.

Protocol

1. Wash 10 mg of amine particles into 10 mM sodium phosphate, pH 7.2, using centrifugation. Resuspend the particles in 1 ml of the same buffer.

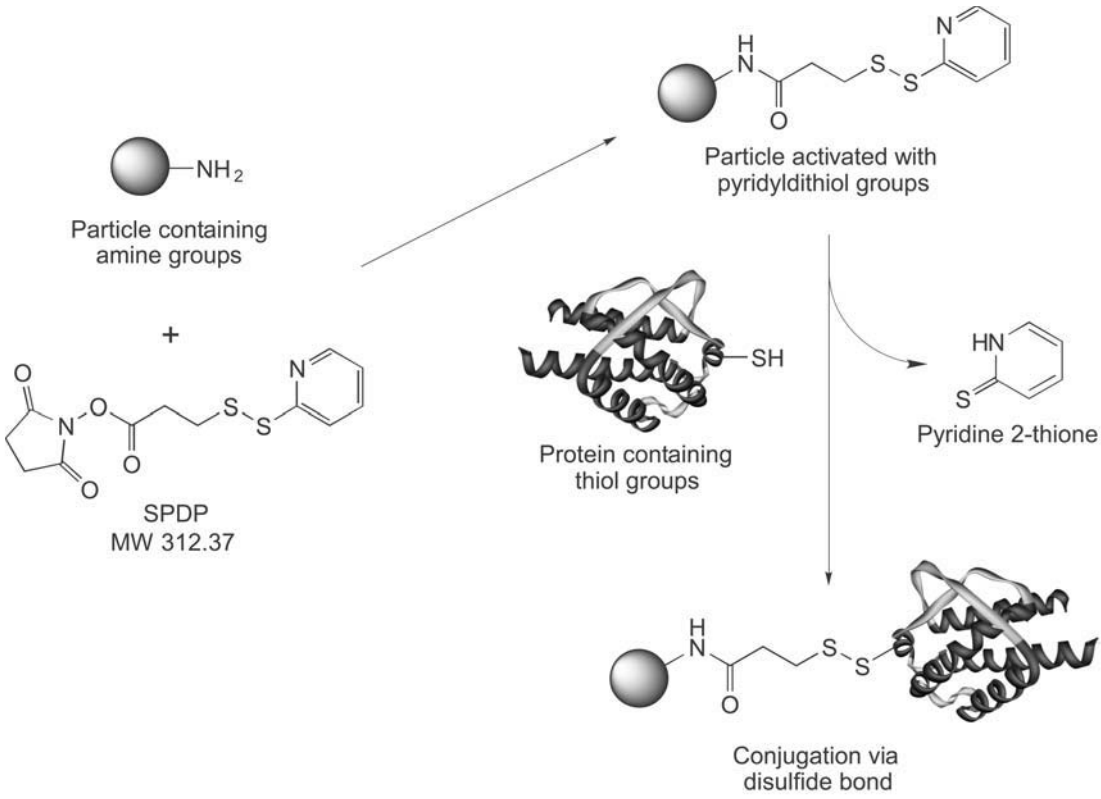


Figure 14.10 The crosslinker SPDP can be reacted with amine particles to create thiol-reactive pyridyl disulfide groups on the surface. Thiol-containing proteins or other thiol molecules can be reacted with these activated particles to result in disulfide linkages, which are reversible by reduction.

2. Dissolve SPDP in dimethylformamide (DMF) at a concentration of 6.2 mg/ml (makes a 20 mM stock solution). Add 50 µl of the SPDP solution to the 1 ml particle suspension and mix to dissolve. *Note*: The small quantity of DMF in a polymeric particle suspension should not affect particle stability, even if the polymer type is susceptible to swelling in pure DMF. Other particle types, such as metallic or silica based, usually are not affected by organic solvent addition, unless their surfaces are non-covalently coated with a dissolvable polymer.
3. React for 30 minutes at room temperature with mixing.
4. Wash particles with coupling buffer at least 3 times using centrifugation and resuspend in the same buffer using a sonic probe to disperse fully the particles.
5. Add 1–10 mg of a protein or antibody containing an available thiol group to the particle suspension. Alternatively, add the protein to be coupled to the particle suspension in an amount equal to 1–10 × molar excess over the calculated monolayer for the protein type to be coupled. The optimal amount of protein to be added should be determined experimentally. Creating thiol groups from disulfides in proteins may be done by using a reducing agent or through the use of a thiolation reagent (Chapter 1, Section 4.1).

6. React with mixing for 2 hours at room temperature. At the completion of the reaction, cysteine may be added at 50 mM to block excess pyridyl disulfide-reactive sites.
7. Remove excess protein and reactants by washing with coupling buffer at least 3 times. Store particles in a suitable buffer containing a preservative.

4.8. NHS-PEG$_n$-Maleimide Coupling to Amine Particles

An alternative method for coupling thiol-containing proteins or antibodies to amine particles is to use a heterobifunctional crosslinker containing an amine-reactive NHS ester at one end and a thiol-reactive maleimide group on the other end (Chapter 5, Section 1). Unlike the pyridyl dithiol reaction with a sulfhydryl as in the SPDP protocol described previously, a maleimide group forms a stable thioether linkage with a sulfhydryl-containing ligand, which is not cleavable by reduction.

A common choice of crosslinker for this type of reaction is sulfo-SMCC (succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate), which has been used extensively for antibody conjugation (Chapter 5, Section 1.3). However, perhaps a better option for particle conjugation is to use a similar crosslinker design, but one in which contains a hydrophilic PEG spacer arm to promote particle hydrophilicity after modification. The modification of an amine particle with a NHS-PEG$_n$-maleimide reagent can create a surface that is essentially coated with PEG spacers (Chapter 18, Section 2). This helps to mask any hydrophobic character that the particle surface may have, while providing terminal thiol-reactive maleimides for coupling ligands (Figure 14.11).

If a NHS-PEG$_n$-maleimide compound is used for this type of activation and coupling, the intermediate maleimide-activated particle should be quickly washed free of excess crosslinker and used to couple ligand immediately. This is due to the fact that the maleimide hydrolyzes in aqueous solution at a higher rate than that of a maleimide on an SMCC-type crosslinker, because of the extreme hydrophilicity of the PEG spacer arm compared to the cyclohexane spacer of SMCC.

NHS-PEG$_n$-maleimide crosslinkers are available in a number of spacer lengths depending on the size of the polymer chain in the PEG component (Chapter 18, Section 2). Long-chain crosslinkers of this type use PEG polymers of molecular weight approximately 2,000–5,000 Da, containing from about 45 to over 100 repeating polyethylene oxide units. These full-length polymers are polydisperse and actually consist of a broad range of polymer lengths, which makes reproducibility of the crosslinker size nearly impossible to achieve. Modifying a particle with this type of polymer diversity causes variability in the length of the crosslinkers displayed on the surface, which may be detrimental for coupling some ligands.

However, shorter, discrete crosslinkers containing PEG spacers of known chain length are now available (Thermo Fisher, Quanta BioDesign). These reagents are designed to contain an exact number of PEG units, typically from between 2 repeating units to 24 repeating units. The following protocol may be used with any of these compounds with the appropriate adjustments in the quantity of crosslinker added to the reaction to take into account differences in molecular weight due to the PEG length. The longer of these discrete NHS-PEG$_n$-maleimide crosslinkers will provide the greatest degree of hydrophilicity after modification of an amine particle surface (see Chapter 18 for additional details on discrete PEG compounds).

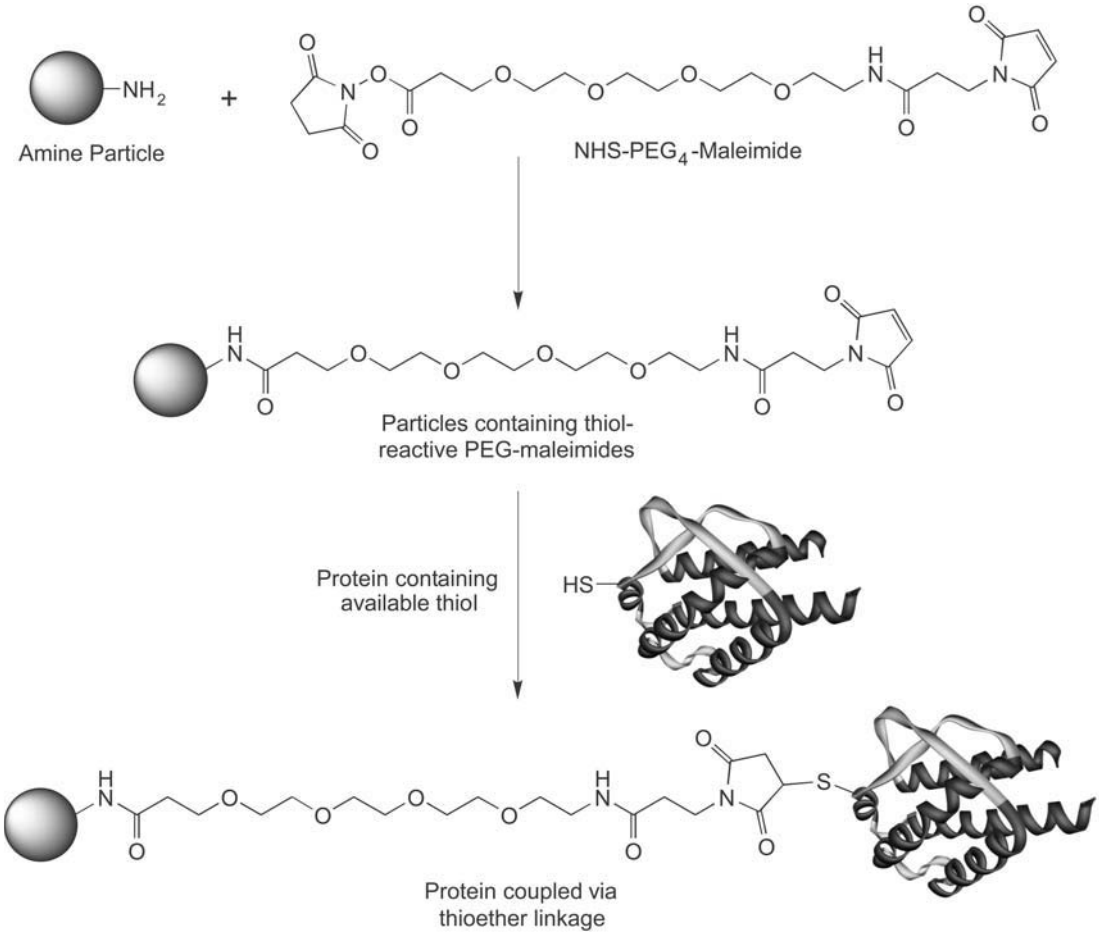


Figure 14.11 The modification of amine-containing particles with NHS-PEG_4-maleimide produces hydrophilic PEG spacers containing terminal thiol-reactive groups. Coupling of thiol-containing proteins then results in the formation of thioether linkages.

Protocol

1. Wash 10 mg of amine particles into 10 mM sodium phosphate, pH 7.2 (coupling buffer) using centrifugation. Resuspend the particles in 1 ml of the same buffer.
2. Dissolve NHS-PEG_6-maleimide (MW 601.6) into dimethyl sulfoxide (DMSO) at a concentration of 20 mM. PEG-type crosslinkers often exist as a thick oily mass, and preparing the solution may involve dissolving an entire vial of the compound into DMSO to determine accurately the required concentration.
3. Add 50 µl of the NHS-PEG_6-maleimide solution to the 1 ml particle suspension and mix thoroughly to dissolve.
4. React for 1 hour at room temperature with mixing.
5. Quickly wash the particles with coupling buffer at least twice using centrifugation.

6. Add 1–10 mg of a protein or antibody containing an available thiol group to the particle suspension. Alternatively, add the protein to be coupled to the particle suspension in an amount equal to 1–10 × molar excess over the calculated monolayer for the protein type to be coupled. The optimal amount of protein to be added should be determined experimentally. Creating thiol groups on proteins or peptides may be done from disulfides by reduction. Alternatively, a thiolation reagent may be used to add thiols to the protein surface for coupling (see the protocols in Chapter 1, Section 4.1).
7. React with mixing for 2 hours at room temperature. At the completion of the reaction, cysteine may be added at 50 mM to block excess maleimide-reactive sites.
8. Remove excess protein and reactants by washing with coupling buffer at least 3 times using centrifugation. Store particles in a suitable buffer containing a preservative.

4.9. Coupling to Hydroxyl Particles

Polymeric particles containing hydroxyl groups often are created from copolymers or composites of pHEMA, frequently with other more rigid polymer cores, such as polystyrene (Figure 14.12). pHEMA particles have surfaces that contain an abundance of primary hydroxyls, which tend to produce favorable hydrophilic surface characteristics (Tauer *et al.*, 2005). The hydroxyls can hydrogen bond with a layer of water molecules in aqueous solution, which forms an interface between individual particles and stabilizes them against aggregation, even in the presence of relatively high salt concentrations. The interaction of biomolecules with pHEMA particles typically lowers nonspecific binding potential compared to particles of more hydrophobic polymer construction. This enhanced hydrophilicity of pHEMA particles translates into a high degree of biocompatibility, which is important for decreasing background in particle-based assays and in preventing denaturation of immobilized proteins on the particle surface.

Although hydroxyls are not spontaneously reactive toward functional groups on biomolecules, they can be activated for covalent coupling by a number of known reaction mechanisms. Most of the reactions that can result in covalent attachment of ligands to hydroxylic or pHEMA particles originated in the development of immobilization technology for affinity chromatography using larger hydroxyl-containing porous beads (for a review, see Hermanson *et al.*, 1992).

Most activation strategies for hydroxylic particles are done under nonaqueous conditions, because the activating agent and the intermediate reactive group typically are susceptible to hydrolysis. A convenient method of activation is to form a reactive carbonyl group on the hydroxyl particle using compounds such as carbonyldiimidazole (CDI; Bethell *et al.*, 1979) or disuccinimidyl carbonate (DSC; Miron and Wilchek, 1993). These activating agents create imidazole carbamates (using CDI) or NHS-carbonates (using DSC) on the particle surface, which then are spontaneously reactive toward amines (see Figures 14.13 and 14.14). After washing away excess activating agent in organic solvent, the particles are centrifuged to remove most solvent and then resuspended in aqueous buffer containing the amine ligand to be coupled (e.g., a protein).

The imidazole carbamate group is more stable to hydrolysis in aqueous buffer than the NHS-carbonate group, which is similar in reactivity to an NHS ester. However, this means that the imidazole carbamate also is slower to react and couple with amines. NHS-carbonate reactions usually go to completion within 1–2 hours at room temperature, whereas imidazole carbamates typically require higher pH conditions and overnight incubations to get maximal yield of ligand coupling.

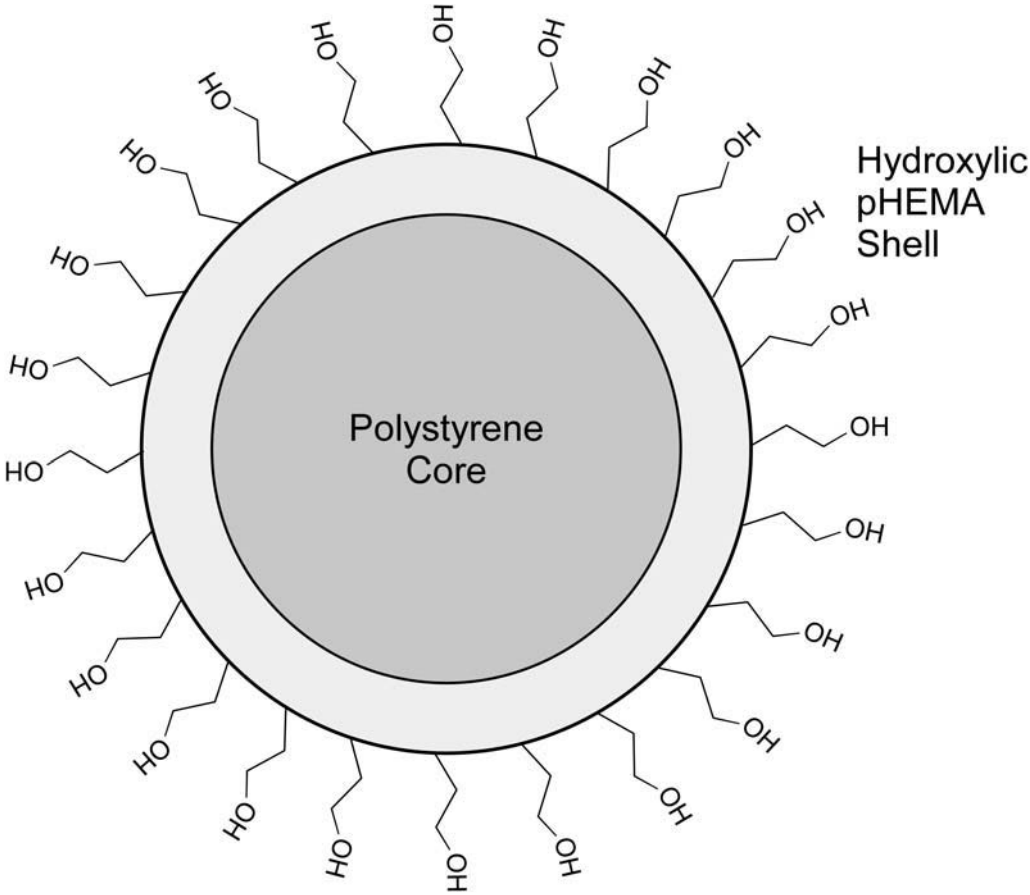


Figure 14.12 A core/shell polymeric particle made of a hydrophobic polystyrene core that is capped with a hydrophilic polyHEMA shell, which contains numerous hydroxyl groups.

The following protocols involve the activation of hydroxylic pHEMA particles in organic solvent using either CDI or DSC and the subsequent coupling of amine-containing molecules using aqueous buffer conditions. Various solvents may be used for the activation step as long as the activation agents are soluble in them and the particles do not get damaged due to solvent exposure. Only water miscible solvents should be used to facilitate exchange into and out of aqueous conditions. However, the solvents should be anhydrous to prevent hydrolysis of the activation agent or the subsequent reactive group formed on the particles.

Protocol Using CDI

This method is derived from that of Bethell *et al.* (1979) and Colvin *et al.* (1988):

1. Solvent exchange pHEMA particles into anhydrous THF using centrifugation and resuspension with sequential exchange into greater percentages of THF in water until the particles

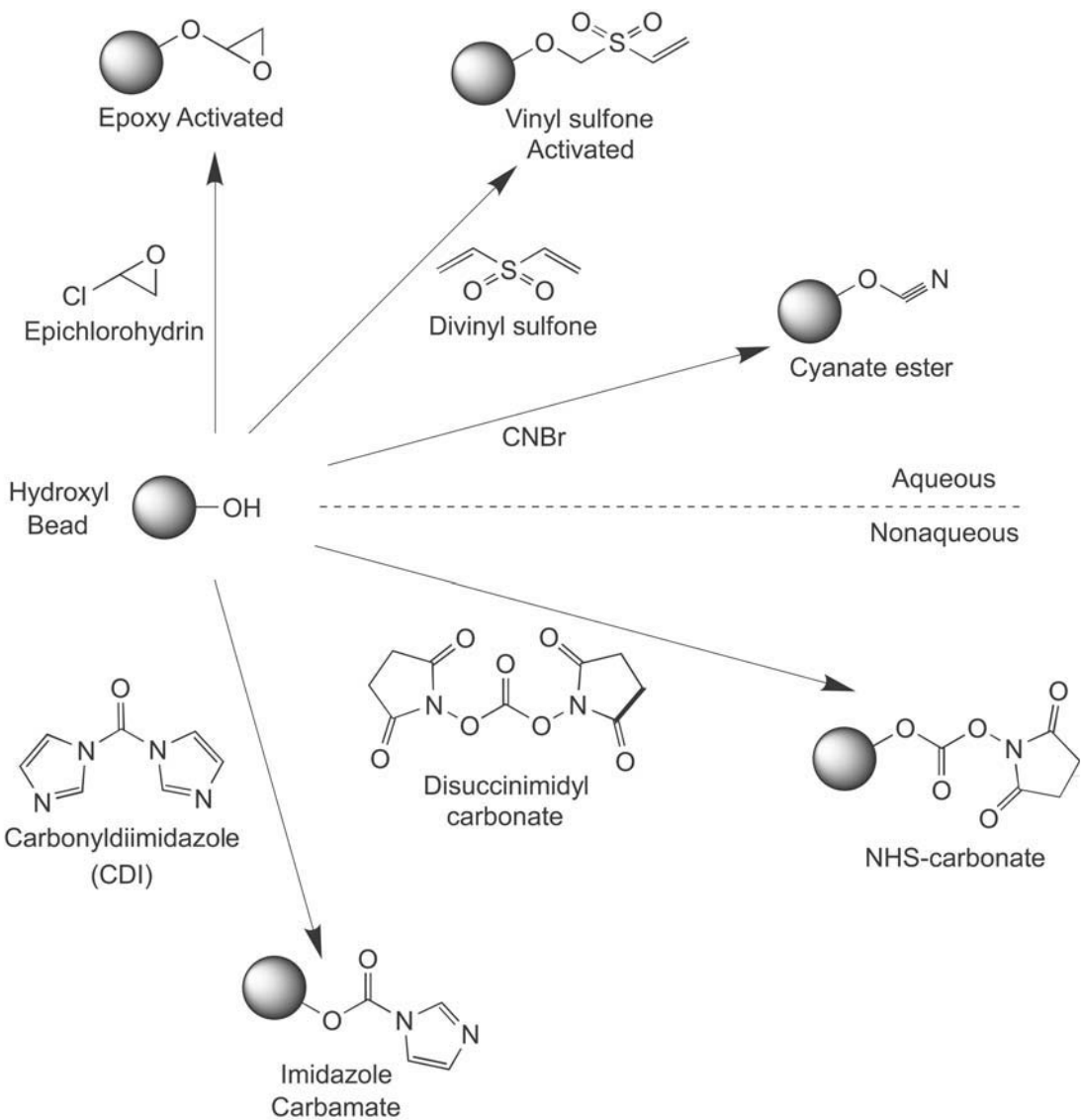


Figure 14.13 Hydroxyl-containing particles can be activated for coupling ligands using a number of strategies, which involve either aqueous or nonaqueous reactions. Epoxy and vinyl sulfone activation procedures provide reactive groups able to couple with amine-, thiol-, or hydroxyl-containing ligands. Cyanogen bromide activation and the CDI and DSC methods provide reactive groups for amine coupling.

are suspended in 100 percent THF. Wash several times with anhydrous THF to eliminate any remaining traces of water, letting the particles agitate in solvent between each centrifugation step. After the final THF wash centrifuge the particles and remove excess solvent (Figure 14.15).

2. Resuspend the particles as a 5 percent suspension in anhydrous THF containing CDI at a concentration of 50 mg/ml (0.3 M).

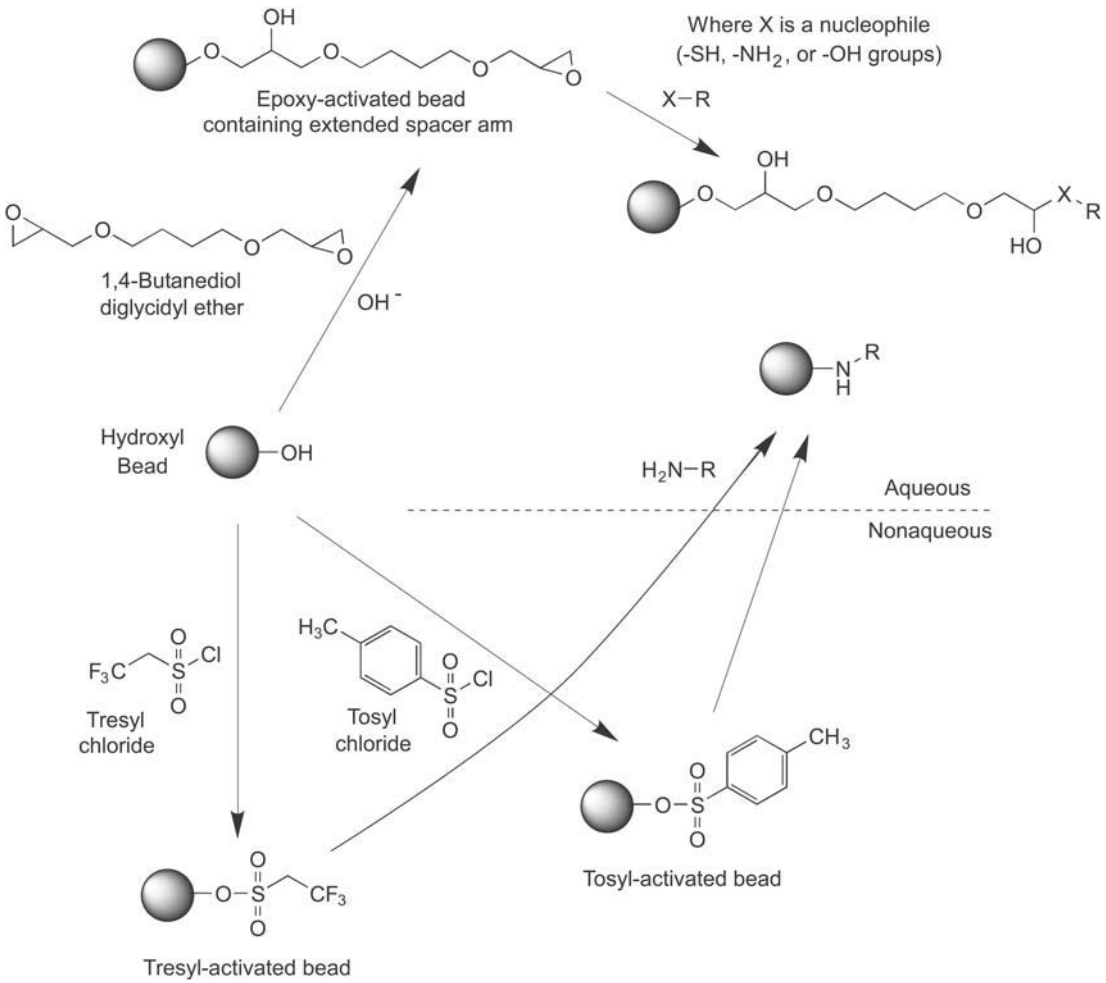


Figure 14.14 Additional hydroxyl-particle activation methods include bis-epoxide modification, tosyl activation, and tresyl activation methods. The tosyl chloride and tresyl chloride activation procedures must be done in dry organic solvent, but the coupling of an amine-containing ligand can be done in either organic solvent or aqueous buffer.

3. React with mixing for 2 hours at room temperature.
4. Wash the activated particles 3 times with anhydrous THF to remove excess CDI and reaction by-products. After the final wash, remove the solvent and perform a quick wash with ice-cold water to remove most traces of solvent in the particle pellet. Finally, resuspend the particles at 10 mg/ml in cold 0.1 M sodium phosphate, pH 8.2, or 0.1 M sodium carbonate, pH 9.5 (coupling buffer). The higher pH coupling buffer will result in greater reactivity of the imidazole carbamate and greater coupling yields for proteins.
5. Add 1–10 mg of a protein or antibody containing an available thiol group to the particle suspension in coupling buffer and mix to dissolve. Alternatively, add the protein to the particle suspension in an amount equal to 1–10 × molar excess over the calculated

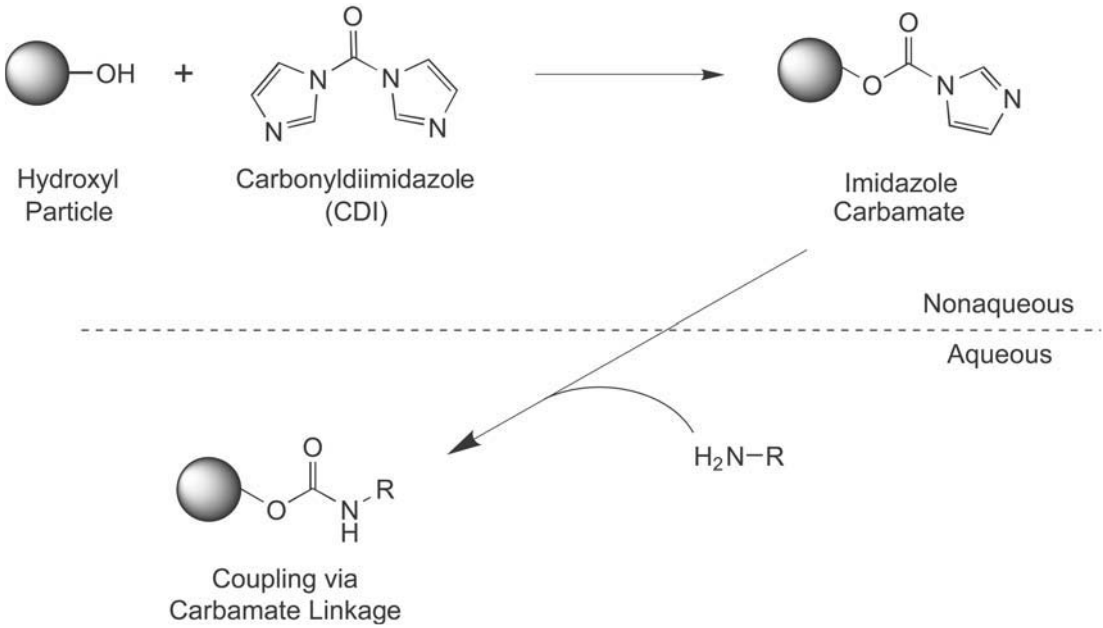


Figure 14.15 CDI can be used to activate hydroxyl-particles in organic solvent and then the intermediate reactive imidazole carbamate brought into aqueous solution for coupling amine-containing ligands.

mono-layer for the protein type to be coupled. The optimal amount of protein to be added should be determined experimentally.

6. React with mixing for at least 18 hours at 4°C. Longer reaction times (24–48 hours) may be necessary when using the pH 8.2 coupling buffer. Room temperature reactions will increase the reaction rate.
7. Add ethanolamine to the particle suspension at a final concentration of 0.1 M to quench any remaining active groups and react with mixing for several hours.
8. Centrifuge and wash the particles at least 3 times with buffer to remove unreacted protein and ethanolamine. Finally, suspend the particles in a suitable storage buffer containing a preservative.

Protocol Using DSC

This method is derived from that of Miron and Wilchek (1993) for the activation of hydroxyl groups on PEG molecules and Wilchek and Miron (1985) for the activation of agarose chromatography beads. Many types of hydroxyl-containing particles may be used in this procedure, provided that the solvent used for the activation step does not deleteriously affect particle integrity (Figure 14.16).

1. Solvent exchange pHEMA particles into anhydrous acetone, dioxane, acetonitrile, THF, or DMF (having very low amine content) using centrifugation and resuspension with sequential exchange into greater percentages of solvent in water until the particles are suspended

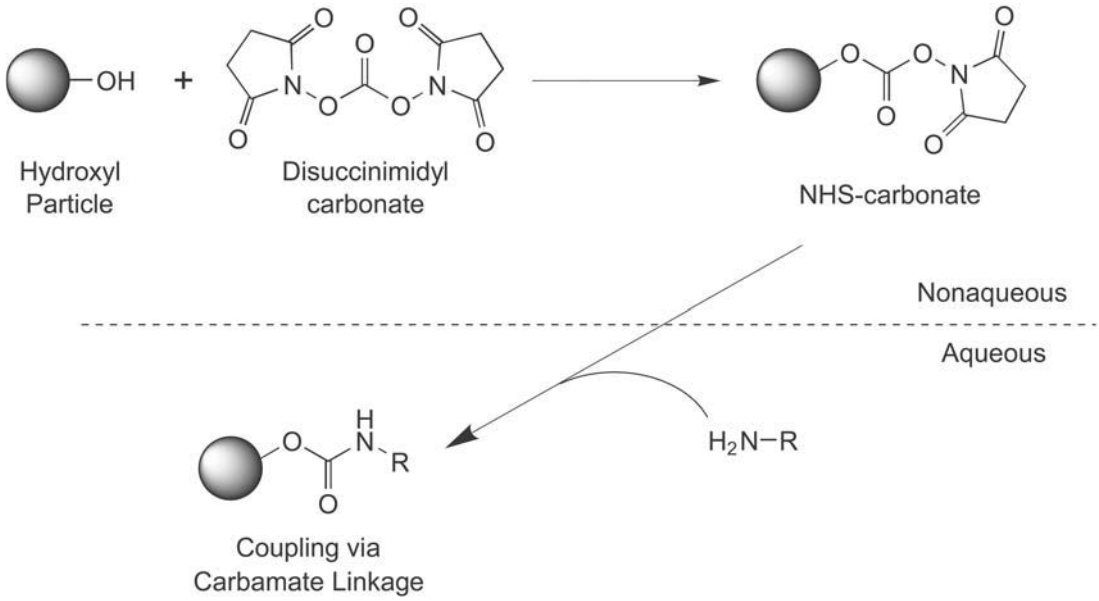


Figure 14.16 DSC can be used to activate hydroxyl-particles to a reactive NHS-carbonate derivative. The subsequent coupling of amine-containing ligands can be done in either organic solvent or aqueous conditions.

in 100 percent organic solvent. Wash several times with anhydrous solvent to eliminate any remaining traces of water, letting the particles agitate in solvent between each centrifugation step. After the final solvent wash, centrifuge the particles and remove excess solvent.

2. Resuspend the particles as a 5 percent suspension in anhydrous solvent containing DSC at a concentration of 50 mg/ml (0.2 M).
3. React with mixing for 2 hours at room temperature.
4. Wash the activated particles 3 times with anhydrous solvent to remove excess DSC and reaction by-products. After the final wash, remove the solvent and perform a quick wash with ice-cold water to remove most traces of solvent in the particle pellet.
5. Immediately resuspend the particles at a concentration of 10 mg/ml in 0.1 M sodium phosphate, pH 7.2 (coupling buffer) containing 1–10 mg of a protein or antibody and mix to dissolve. Alternatively, add the protein to the particle suspension in an amount equal to 1–10 × molar excess over the calculated monolayer for the protein type to be coupled. The optimal level of protein to be added should be determined experimentally.
6. React with mixing for at least 2 hours at room temperature or twice as long at 4°C.
7. Add ethanolamine to the particle suspension at a final concentration of 0.1 M to quench any remaining active groups and react with mixing for 1 hour. Other amine-containing quenchers may be used, too, such as Tris buffer. *Note*: DSC-activated sites on the particles that completely hydrolyze will revert back to the original hydroxyls.
8. Centrifuge and wash the particles at least 3 times with buffer to remove unreacted protein and quenching agent. Finally, suspend the particles in a suitable storage buffer containing a preservative.

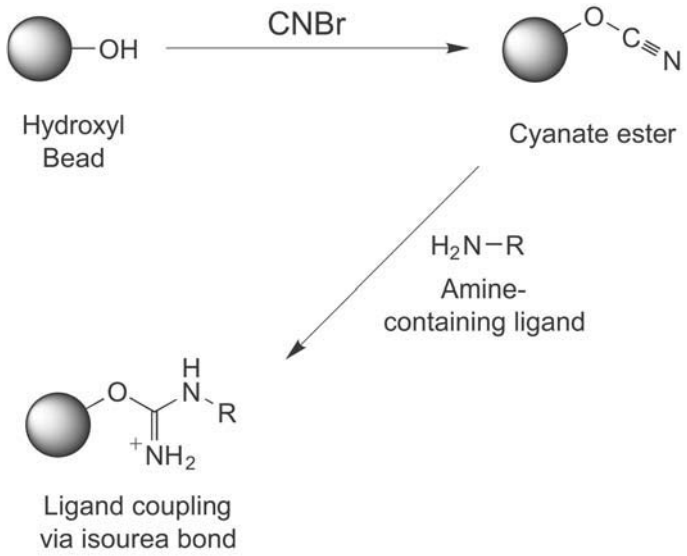


Figure 14.17 Cyanogen bromide can be used to activate a hydroxyl-particle to a reactive cyanate ester, which then can be used to couple amine-containing ligands.

Protocol Using Cyanogen Bromide

Cyanogen bromide can be used to activate hydroxyl groups on particles to create reactive cyanate esters, which then can be coupled to amine-containing ligands to form an isourea bond (Figure 14.17). CNBr activation also can produce cyclic imidocarbonate groups, which are less reactive than the cyanate ester, but can form imidocarbonate bonds. The exact reactive species formed by the reaction is dependent on the structure of the hydroxylic support being activated (Kohn and Wilchek, 1982).

Unlike the previous methods for activation of hydroxyls on particles, cyanogen bromide is used under aqueous conditions, thus eliminating the need for organic solvents. This method originated in the activation and coupling of ligands to agarose supports for affinity chromatography (Axen *et al.*, 1967), and it subsequently was used to couple antibodies to hydroxyl-containing latex particles (Yen *et al.*, 1979) and more recently to immobilize protein A onto pHEMA particles (Denizli *et al.*, 1995).

Cyanogen bromide is an extremely toxic chemical and should be used only in a well-ventilated fume hood using the appropriate personal protective gear. The following protocol is based on the method of March *et al.* (1974), as recommended by Bang's Laboratories.

All operations should be performed in a fume hood:

1. Wash 100 mg of hydroxyl-containing particles 2 times using centrifugation with 0.1 M sodium carbonate, pH 8.5 (coupling buffer). After the second wash, resuspend the particles at 10 mg/ml in 2 M sodium carbonate (activation buffer; no pH adjustment necessary).
2. Dissolve an amount of protein in 10 ml of coupling buffer equal to 1–10 × excess over the calculated monolayer for the type of particles being used.

3. In a fume hood, dissolve 1 g of cyanogen bromide in 0.5 ml of acetonitrile (highly toxic!).
4. Add a drop of the cyanogen bromide solution at a time to the particle suspension with constant mixing at room temperature. The entire solution should be added to the particles over the course of about 10 seconds.
5. Activate the particles with mixing for exactly 2 minutes at room temperature.
6. Quickly wash the particles with ice-cold deionized water and then with a volume of cold coupling buffer. Resuspend the particles in the protein solution prepared in step 2.
7. React for 24 hours at 4°C with mixing.
8. Wash the particles with coupling buffer and block excess reactive groups by resuspending in 50 mM ethanolamine, pH 9.0. React for 1 hour at room temperature with mixing.
9. Thoroughly wash the particles with storage buffer (e.g., PBS, pH 7.5, or other suitable buffer) and resuspend them at 10 mg/ml in storage buffer containing a preservative.

4.10. Coupling to Hydrazide Particles

Hydrazide particles can be made from carboxylate particles by modification with a bis-hydrazide compound using the carbodiimide reaction with EDC. Suitable bifunctional hydrazides include the small carbohydrazide compound or the longer adipic dihydrazide (Chapter 4, Section 8). A bis-hydrazide compound is reacted with a carboxylate particle population in large excess to prevent particle polymerization during the reaction. The resultant hydrazide particles may be used to couple to carbonyl-containing ligands, such as carbohydrates or glycans at their reducing ends or after the formation of aldehydes on carbohydrates using oxidation with sodium periodate (Chapter 1, Section 4.4).

Perhaps a better design for a bis-hydrazide compound to modify carboxylate particles would include a short PEG spacer arm between the two hydrazide groups. This type of linker would result in a hydrophilic surface due to the presence of the PEG spacers, while providing the terminal hydrazide functionality necessary for coupling to carbonyl compounds. Unfortunately, this type of compound is not currently available, so the aliphatic bis-hydrazides are the only choice.

Another route to the formation of a hydrazide on a surface is to use an aldehyde-containing particle (such as HEMA/acrolein copolymers) and subsequently modify the aldehydes to form hydrazone linkages with bis-hydrazide compounds, which then can be stabilized by reduction with sodium cyanoborohydride (Chapter 2, Section 5). The resulting derivative contains terminal hydrazides for immobilization of carbonyl ligands (see Figure 14.18).

Hydrazide-containing particles provide functional groups for the coupling of aldehyde- or ketone-containing ligands through a dehydration reaction to form hydrazone linkages (a type of Schiff base). However, a single hydrazone bond between a ligand and the particle surface may not provide enough stability to prevent leaching of ligand due to hydrolysis. There are two routes to overcome this instability: (1) reduce the hydrazone linkage using sodium cyanoborohydride or (2) create multiple hydrazone linkages between the ligand and the particle surface. Multi-site attachment provides sufficient ligand stability, because not all the hydrazones will hydrolyze simultaneously to release ligand, and when one hydrazone bond breaks, it will have enough time to reform before the other hydrazones hydrolyze. Thus, glycosylated proteins coupled after oxidation to hydrazide particles most likely will be stable due to the presence of

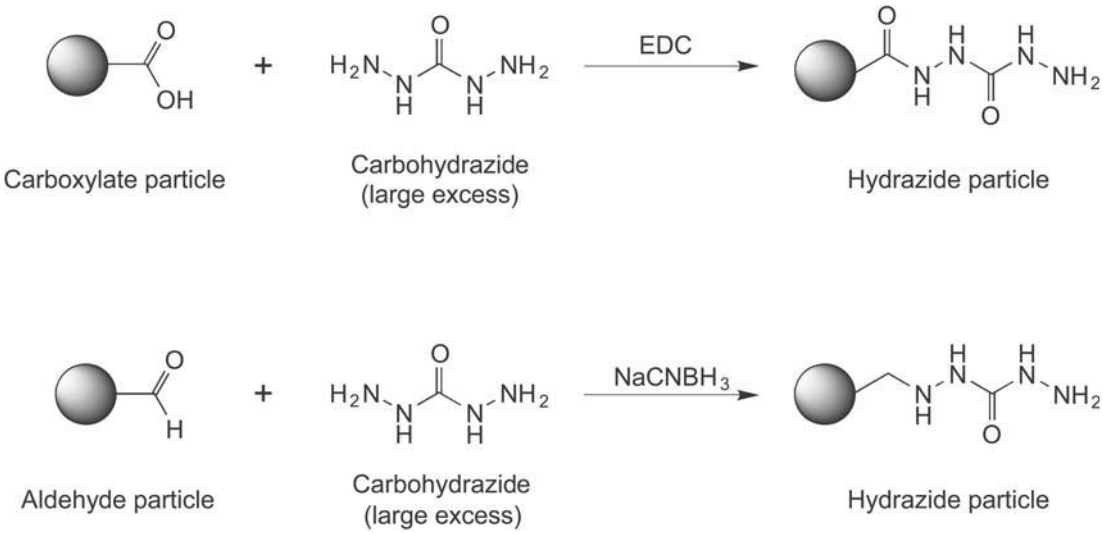


Figure 14.18 Carboxylate-particles or aldehyde-particles can be modified with the carbohydrazide in excess to create a hydrazide-particle that can be used to couple with aldehyde-containing molecules.

more than one aldehyde group, but small ligands containing only a single carbonyl group probably should be treated with cyanoborohydride to stabilize the hydrazone bond.

The reactions involved with coupling carbonyl-containing ligands to hydrazide particles originated with the activation and coupling chemistry associated with the preparation of affinity chromatography supports (O'Shannessy and Wilchek, 1990). The application of this strategy to hydrazide-containing microparticles or nanoparticles is straightforward and will work well so long as accommodation is given to particle stability during the process. Using this method, a broad range of carbonyl-containing ligands can be coupled, such as reducing sugars or carbohydrates containing a reducing end, glycans after release from proteins, glycoproteins and other glycoconjugates, and small organic compounds containing an aldehyde or ketone group (Figure 14.19). Horak *et al.* (1999) used hydrazide-pHEMA particles to couple oxidized horseradish peroxidase (HRP) in good yield and retention of enzymatic activity.

The following protocol describes the oxidation of carbohydrate (glycans) on antibody molecules to form aldehydes and the subsequent coupling to hydrazide particles.

Protocol

1. Dissolve the antibody to be coupled in 10 mM sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 10 mg/ml. The antibody must be glycosylated to work in this procedure.
2. Prepare a solution of 0.1 M sodium periodate in water. Protect from light.
3. With mixing, add 0.1 ml of the periodate solution to each ml of the antibody solution.
4. React for 30 minutes at room temperature, protected from light.
5. The reaction may be quenched by the addition of 0.1 ml of glycerol per ml of reaction or by the addition of sodium bisulfite to a final concentration of 10 mM.

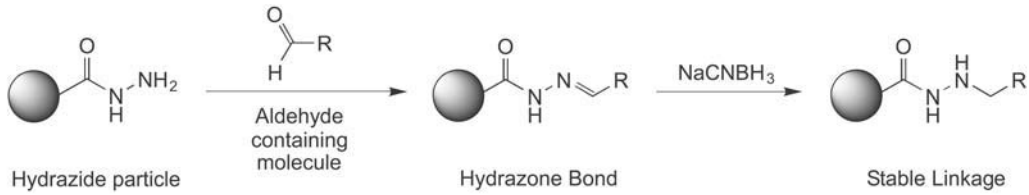


Figure 14.19 Aldehyde-containing molecules, such as periodate-oxidized carbohydrates or glycoproteins, can be coupled to hydrazide-particles to form a hydrazone bond. This bond can be further stabilized by reduction with sodium cyanoborohydride.

6. Remove excess reactants from the reaction mixture using size exclusion chromatography on a column of Sephadex G25 (or equivalent).
7. Wash 100 mg of hydrazide particles 2 times with 10 ml of 10 mM sodium phosphate, 0.15 M NaCl, pH 7.5 (coupling buffer) using centrifugation.
8. After the final wash, resuspend the particles at a concentration of 10 mg/ml in coupling buffer and add an appropriate amount of the solution from step 6, which contains the purified, oxidized antibody. The amount of oxidized antibody to add to the particles should be about 1–10 × over the amount of the calculated monolayer for the particle type used. (*Note*: For 100 mg of 1 μm hydrazide particles, a monolayer equivalent of antibody will be about 1.5 mg, so the total amount added should be in the range of 1.5–15 mg for a 1–10 × excess).
9. React with mixing for at least 6 hours at room temperature or overnight at 4°C.
10. Wash the beads at least several times with coupling buffer and resuspend in the same buffer containing 0.05–0.1 percent of a blocking molecule (such as BSA, gelatin, nonfat dried milk, PEG, PVP, etc.). The best blocking agent may be found by experimentation and testing of the antibody-coupled particles in the intended application.
11. Wash the particles several times with coupling buffer and store in an appropriate buffer containing a preservative at 4°C.

4.11. Coupling to Epoxy Particles

Polymeric particles containing epoxide groups can be used to couple thiol-, amino-, or hydroxyl-containing ligands via a ring-opening reaction facilitated under basic conditions. This reactive group can be used to couple proteins, nucleic acids, sugars and carbohydrates, and other organic molecules containing these functionalities. Epoxide groups can be introduced into polymeric particles through free-radical copolymerization with oxirane-containing vinyl monomers, such as allyl glycidyl ether, or they may be introduced by surface modification using a bis-epoxide compound, such as 1,4-butanediol diglycidyl ether (Sundberg and Porath, 1974). Epoxy activation has been used extensively to immobilize affinity ligands onto porous beaded chromatography supports, and it can be used with equal success to couple ligands to microparticles and nanoparticles.

Epoxide-containing particles can be used to couple thiol-containing ligands at slightly basic pH (pH 7.5–8.5), amine-containing ligands at higher pH values (pH 9–11), and hydroxyl-containing ligands at very high alkaline conditions (pH > 11). The following protocol can be

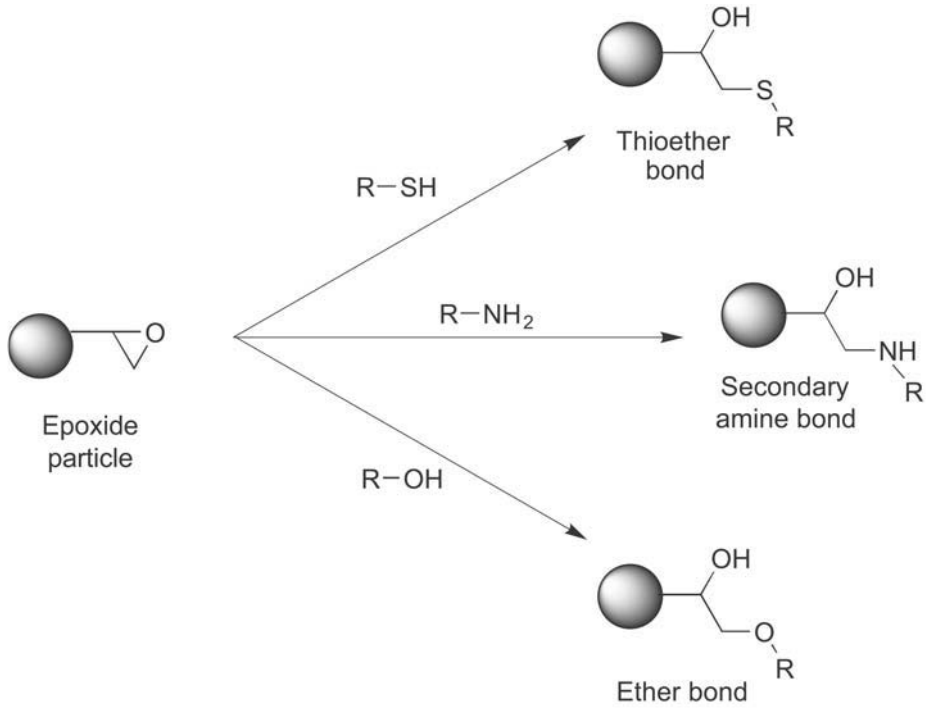


Figure 14.20 Particles containing reactive epoxy groups can be coupled with amine-, thiol-, or hydroxyl-containing molecules.

used to couple thiol-, amine-, or hydroxyl-containing ligands with the proper pH adjustment of the carbonate coupling buffer (Figure 14.20).

Protocol

1. Wash 100 mg of epoxy particles with coupling buffer (i.e., 0.1 M sodium carbonate, pH 10 for coupling amine-containing ligands). Use higher pH conditions if coupling hydroxylic molecules and lower pH for coupling thiol-containing ligands. Suspend the particles at a 5 percent solution in coupling buffer.
2. With mixing, add to the particle suspension a quantity of ligand dissolved in coupling buffer in an amount that represents a 1–10 × excess over the molar quantity of epoxide groups present on the particles.
3. React at room temperature (for sensitive ligands) or at 45–60°C (for more stable ligands) for at least 20 hours with mixing.
4. Block excess epoxy groups by the addition of cysteine to a final concentration of 50 mM. Other small molecules can be used, provided they will efficiently react with the excess epoxides and not result in a modification that could interfere with the subsequent use of the particles. Continue the reaction with mixing for at least 2 hours.
5. Wash the particles thoroughly with coupling buffer and then into a more moderate pH storage buffer containing a preservative.

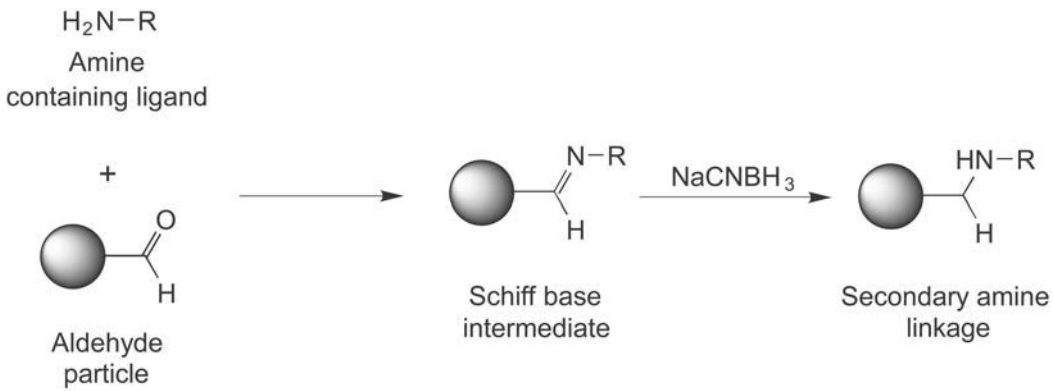


Figure 14.21 Aldehyde-particles can be reacted with amine-containing proteins or other molecules to form intermediate Schiff bases, which can be stabilized by reduction with sodium cyanoborohydride.

4.12. Coupling to Aldehyde Particles

Polymeric particles containing aldehydes are produced by two general routes: copolymers containing an aldehyde monomer (e.g., acrolein) or through periodate oxidation of diols incorporated onto the surface of particles. An example of copolymer aldehyde particles are HEMA/acrolein derivatives (Kumakura and Kaetsu, 1984; Chang *et al.*, 1986; Colvin *et al.*, 1988), which are hydrophilic due to the large number of hydroxyl groups and provide aldehydes for covalent attachment of amine-containing ligands. Epoxy particles also can be used to create surface aldehydes by opening up the epoxide ring by acid hydrolysis and oxidation of the resultant diols by sodium periodate (Schiel *et al.*, 2006).

Aldehyde particles are spontaneously reactive with hydrazine or hydrazide derivatives, forming hydrazone linkages upon Schiff base formation. Reactions with amine-containing molecules, such as proteins, can be done through a reductive amination process using sodium cyanoborohydride (Figure 14.21).

Protocol

1. Wash 10 mg of aldehyde particles 3 times with 10 mM sodium phosphate, pH 7.4 (coupling buffer). Buffers of higher pH value (i.e., carbonate buffer at pH 10) will result in more efficient Schiff base formation with amine-containing molecules than neutral pH conditions.
2. After the final wash, suspend the particles at 5–10 mg/ml in coupling buffer and add a protein to be coupled to the particle suspension in an amount equal to 1–10 × molar excess over the calculated monolayer for the protein type to be coupled. (*Note*: It takes about 18 mg of BSA or 15 mg of IgG to saturate 1 g of 1 μm particles, and more protein if the particles are smaller.) Mix thoroughly to dissolve. Low concentrations of protein may result in particle aggregation, because a single protein molecule can react and bridge more than one particle.
3. Incubate with mixing for 2–4 hours at room temperature.

4. Add to the particle suspension a quantity of sodium cyanoborohydride in water to bring the final concentration up to 10 mM. If a high pH buffer was used for the initial incubation between the particles and the protein, perform a quick wash with 10 mM sodium phosphate, pH 7.4, to bring the pH down to a point in which the reducing agent is active. Mix for 30 minutes at room temperature. The reducing agent will convert all the resultant Schiff bases into stable secondary amine linkages.
5. Add to the particle suspension a quenching molecule (such as glycine, ethanolamine, or Tris) to give a final concentration of 0.2 M. The blocking agent will couple to any remaining aldehyde-reactive sites.
6. Remove excess protein and reactants by washing with coupling buffer at least 3 times using centrifugation. Store particles in a suitable buffer containing a preservative.

5. Silica Particles

The use of silica particles in bioapplications began with the publication by Stöber *et al.* in 1968 on the preparation of monodisperse nanoparticles and microparticles from a silica alkoxide monomer (e.g., tetraethyl orthosilicate or TEOS). Subsequently, in the 1970s, silane modification techniques provided silica surface treatments that eliminated the nonspecific binding potential of raw silica for biomolecules (Regnier and Noel, 1976). Derivatization of silica with hydrophilic, hydroxylic silane compounds thoroughly passivated the surface and made possible the use of both porous and nonporous silica particles in all areas of bioapplications (Schiel *et al.*, 2006).

The modification of silica particles to provide sites for coupling affinity ligands can be done similarly through covalent derivatization of the surface with a functional silane containing a side-chain-reactive group or functional group (see Chapter 13). For instance, reaction of a silica particle with 3-aminopropyltriethoxysilane (APTS) under the appropriate conditions coats the surface with primary amino groups for conjugation with electrophilic groups. Reagents containing alkoxy silane groups can condense, particularly after hydrolysis to create reactive silanols, with the silanol —OH groups on standard silica particles to form stable siloxane linkages (or oxane bond; Figure 14.22). This reaction typically is catalyzed by heat or through the use of at least a partially aqueous environment, which forms the requisite silanols from the alkoxy groups on the silane compound. Unlike other alkoxy silanes, trimethoxy silane compounds can react directly with particle silanols without the need for high-temperature conditions or hydrolysis to form siloxane linkages. Choice of the appropriate silane functional group for surface modification can provide a broad range of silica particle properties for subsequent coupling of biomolecules or other affinity ligands (for review see VanDerVoort *et al.*, 1996).

Silica particles have some advantages over polymer particles. They have a higher density than polymeric particles (1.96 g/cm^3 versus 1.05 g/cm^3) and thus they can be washed using centrifugation, even when working with nanometer-sized particles. Typically, silica particles as small as 30–40 nm still can be separated from suspension using a benchtop microfuge, making handling and processing of silica particles potentially much simpler than polymeric particles of the same size.

Another advantage of using inorganic silica particles over polymer particles is that they don't shrink or swell when exposed to aqueous or nonaqueous environments. In the case of

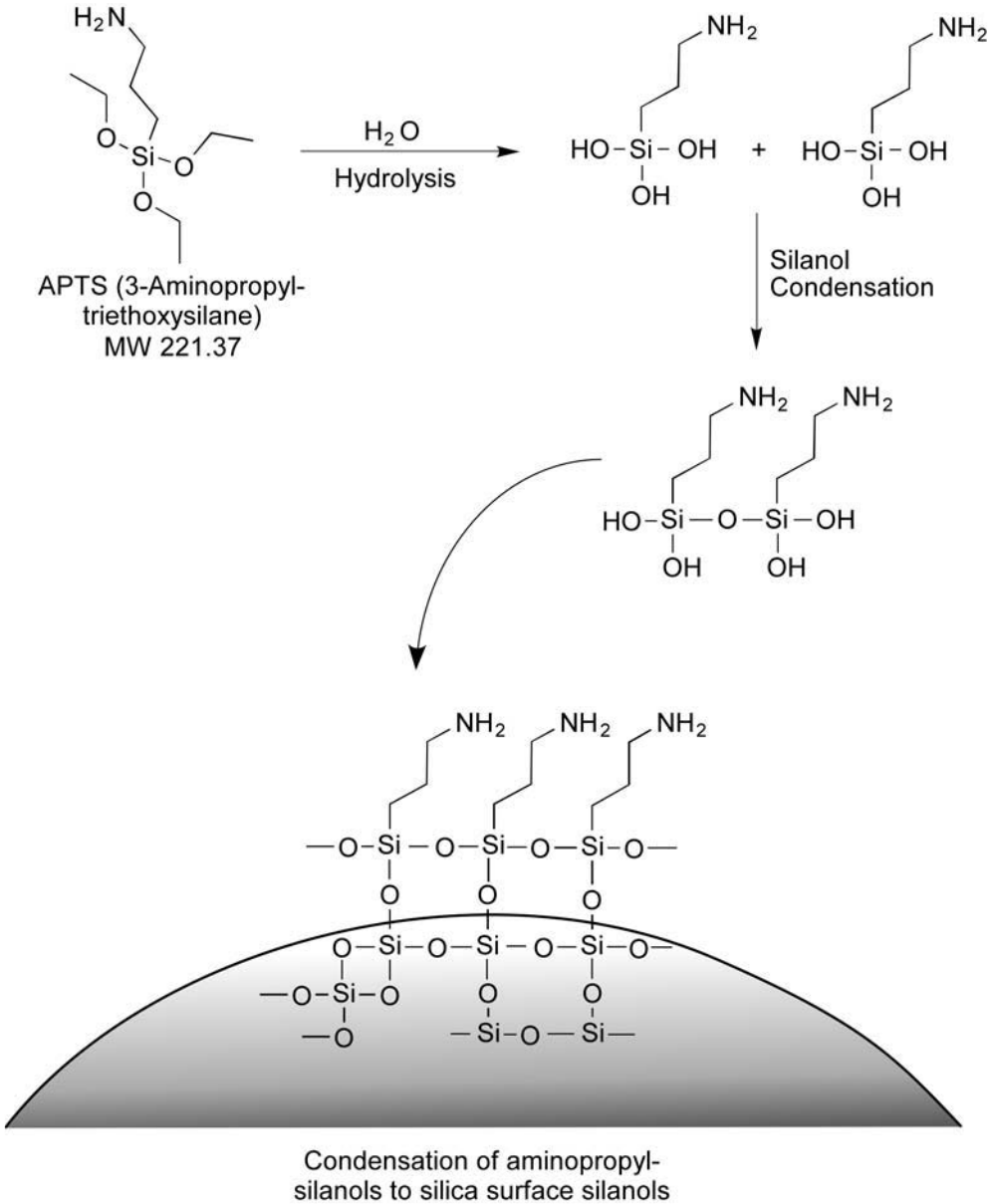


Figure 14.22 Silica particles can be functionalized to contain amine group by reaction with APTS.

silica, nonaqueous solvents may be used for activation reactions without concern that softening or dissolving of polymeric structures will damage the particles, as silica does not dissolve in organic solvent environments.

A potential disadvantage of silica-based particles, however, is the tendency for the siloxane linkages between silicon atoms within the particle to dissolve hydrolytically, especially under

alkaline conditions above pH 8.0. Surface treatment with organosilane derivatives can stabilize particles to hydrolysis, but long-term exposure to highly alkaline environments still should be avoided. It is also recommended that at least 0.05 M NaCl be maintained to increase stability of the particles in aqueous environments. The best stability for silica is obtained at neutral or acidic pH conditions containing a low concentration of salt.

5.1. Fluorescent Silica Particles

Silica particles have been exploited in virtually every assay or detection strategy that polymer particles have been used in for bioapplication purposes. Recently, fluorescent dye-doped silica nanoparticles have been developed by a number of groups that have similar fluorescence characteristics to quantum dot nanocrystals (Chapter 9, Section 10). Fluorescent silica nanoparticles can be synthesized less expensively than quantum dots due to the fact that the silica particles incorporate standard organic dyes (Ow *et al.*, 2005; Wang *et al.*, 2006) and are not dependent on making reproducible populations of semiconductor particles with precise diameters to tune emission wavelengths.

The preparation of fluorescent silica particles can be done using a number of strategies. Santra *et al.* (2001) describe a water-in-oil emulsion using detergent-mediated reverse micelle formation and controlled hydrolysis of TEOS to create mono-disperse silica nanoparticles (see also Arriagada and Osseo-Asare, 1995). Adding the water-soluble fluorescent dye, tris(2,2′-bipyridyl) dichlororuthenium (II) hexahydrate ($Ru(II)bpy_3^{2+}$) to this emulsion resulted in dye molecules being entrapped within the silica particle structure as it formed (Figure 14.23) (also see Chapter 28, Section 4.2 for additional properties of $Ru(II)bpy_3^{2+}$). Subsequent functionalization of the surface with silane derivatives can be done to facilitate ligand immobilization.

These $Ru(II)bpy_3^{2+}$ fluorescent silica nanoparticles were used to detect single bacterial cells using antibodies conjugated to the surface after functionalization with trimethoxysilylpropyldiethylenetriamine followed by succinylation to create carboxylates. Specific antibody molecules against *E. coli* O157 then were coupled to this modified fluorescent particle using the carbodiimide method with EDC and NHS (Zhao *et al.*, 2004).

Another type of fluorescent silica particle was formed from silica bubbles created on the surface of gold nanoparticles (Liz-Marzan *et al.*, 1996; Makarova *et al.*, 1999). Fluorescein isothiocyanate (FITC) was adsorbed onto the gold nanoparticle surface and then reacted with 3-aminopropyltrimethoxy silane. The isothiocyanate groups on the adsorbed dye molecules coupled to the amine groups on the silane as it polymerized on the gold surface, effectively forming a silica shell around the gold particle. The gold core then was dissolved by reaction with cyanide ions to leave behind the silica nanobubbles filled with water, which also left the fluorescent molecules attached on the inner surface (Figure 14.24).

Fluorescent silica nanoparticles, called FloDots, were created by Yao *et al.* (2006) by two synthetic routes. Hydrophilic particles were produced using a reverse micro-emulsion process, wherein detergent micelles formed in a water-in-oil system form discrete nanodroplets in which the silica particles are formed. The addition of water-soluble fluorescent dyes resulted in the entrapment of dye molecules in the silica nanoparticle. In an alternative method, dye molecules were entrapped in silica using the Stöber process, which typically results in hydrophobic particles. Either process resulted in luminescent particles that then can be surface modified with

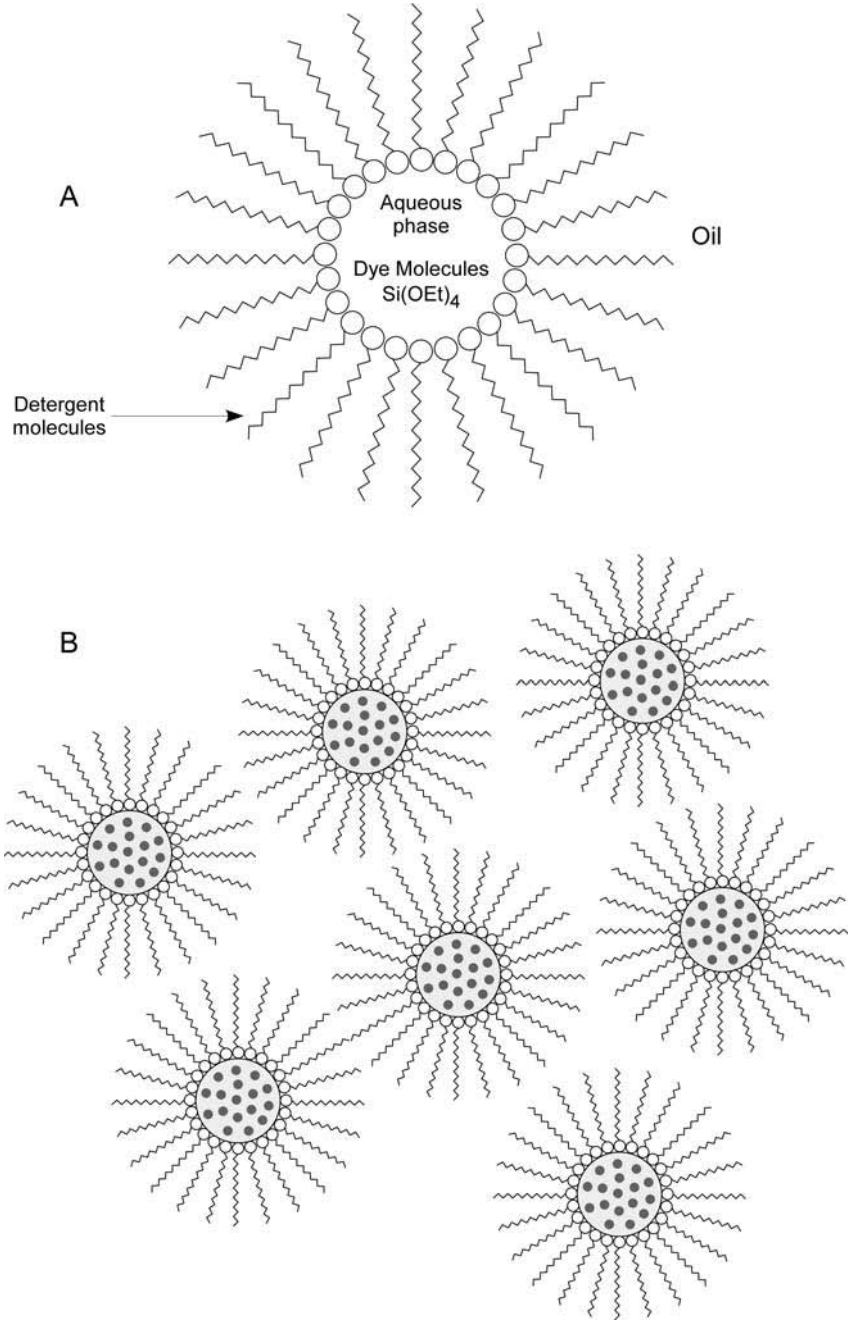


Figure 14.23 Silica nanoparticles containing fluorescent dye molecules can be prepared using a reverse micelle suspension process: (a) The water-in-oil emulsion is formed with the aqueous phase droplets containing TEOS and dye molecules in detergent. (b) The final particles contain entrapped dye within the silica particle matrix, creating highly fluorescent particles.

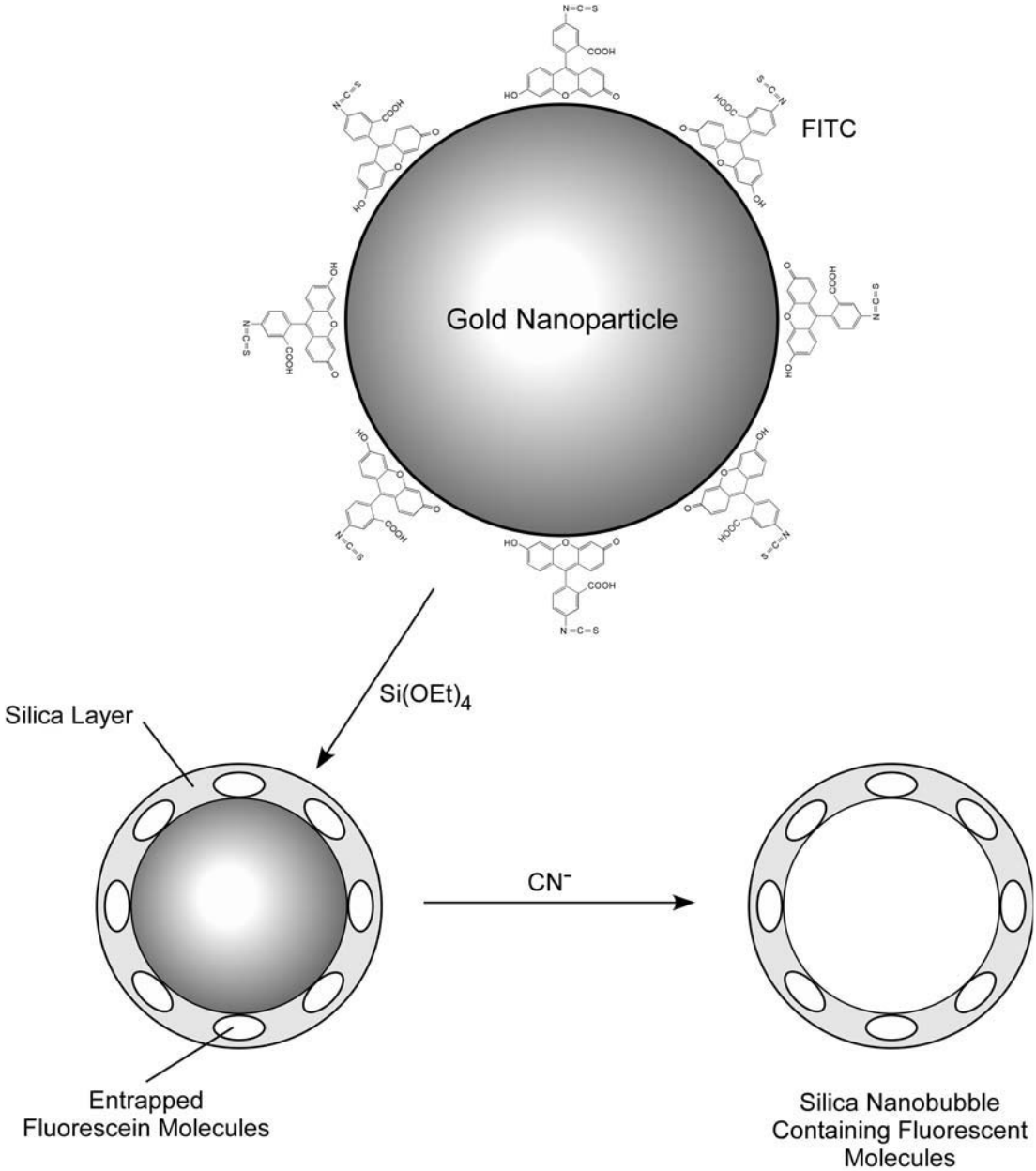


Figure 14.24 Fluorescent silica nanobubbles have been created using gold nanoparticle seeds that initially are coated by adsorption with a fluorescent dye. The particles then are capped by a layer of silica by polymerizing TEOS and entrapping the dye molecules within it. Finally, the gold core is dissolved by reaction with cyanide, leaving behind hollow fluorescent silica nanobubbles.

functional silanes to contain appropriate functionalities for coupling to affinity ligands or biomolecules. $Ru(II)bpy_3^{2+}$ or standard organic fluorescent dyes can be incorporated into such silica particles with high efficiency. In one example, a 70 nm particle containing $Ru(II)bpy_3^{2+}$ was found to be equal in fluorescence intensity to 39 quantum dots having an emission at 605 nm (Yao *et al.*, 2006). Another silica nanoparticle prepared using a rhodamine dye contained about 1,290 molecules of the dye entrapped within each particle, which produced intensely fluorescent labels.

In a different method of producing dye-doped silica particles, van Blaaderen and Vrij (1992) and Verhaegh and van Blaaderen (1994) developed a method to covalently link amine-reactive dyes to APTS and then polymerize the resultant conjugate with TEOS in mixtures of ammonia, water, and ethanol to form fluorescent "organosilica" spheres. The dye molecules could be dispersed throughout the entire particle, contained in the core, or within discrete spherical regions within the particles. Either hydrophilic or hydrophobic particles could be created, depending on the surface treatment used subsequent to particle formation. Fluorescent particles consisting of either fluorescein or rhodamine dyes made by this method were shown to be susceptible to photobleaching similar to the organic dyes in solution.

The following protocol is based on the creation of fluorescent silica core/shell particles using the method of van Blaaderen and Vrij (1992).

Protocol

Formation of dye-silica core particles:

1. React APTS (11.5 mg) with FITC (10.6 mg) in anhydrous ethanol (1 ml) with mixing for 12 hours (protect from light). The use of anhydrous conditions will prevent the hydrolysis of the APTS alkoxy groups, which would cause premature condensation.
2. Prepare a solution consisting of 75 ml of ethanol containing 8.5 ml of ammonia.
3. To the stirring ethanol/ammonia solution, add 3.3 ml of TEOS along with the completed reaction solution from step 1, which is now the conjugate of fluorescein and APTS linked through the amino group on the alkyl silane.
4. The reaction is allowed to continue for 24 hours with slow stirring.
5. Wash the resultant core particles twice with the ethanol/ammonia solution using centrifugation.

Formation of the silica shell:

1. Dilute the particles to 1.2 l using a 10:1 mixture of ethanol:ammonia. Add 28 ml of TEOS to the particle suspension and mix to dissolve.
2. React for 24 hours with slow mixing.
3. Wash the particles with the ethanol/ammonia solution several times using centrifugation. Finally, wash the particles into the desired solution (ethanolic or aqueous) without ammonia present to prevent silica hydrolysis upon storage.

Ow *et al.* (2005) developed an improved method of incorporating fluorescent molecules into silica particles using a modified Stöber synthesis, which resulted in both enhanced fluorescence and photostability of the encapsulated dyes. In this two-stage procedure, reactive organic dyes

first are conjugated to a silane derivative and condensed to form a polysiloxane-dye-rich nanoparticle core structure. No TEOS is added at this point. These dyed core nanoparticles then are used as seed for the addition of silica sol–gel monomers to condense around the core and create a silica network (shell) around the dye-rich core (Figure 14.25). Nyffenegger *et al.* (1993) used a similar process to form fluorescein particles by first coupling FITC to 3-aminopropyltrimethoxy silane to form a thiourea derivative, and then condensing the dye silane derivative with tetramethoxy silane to form the dyed silica particles.

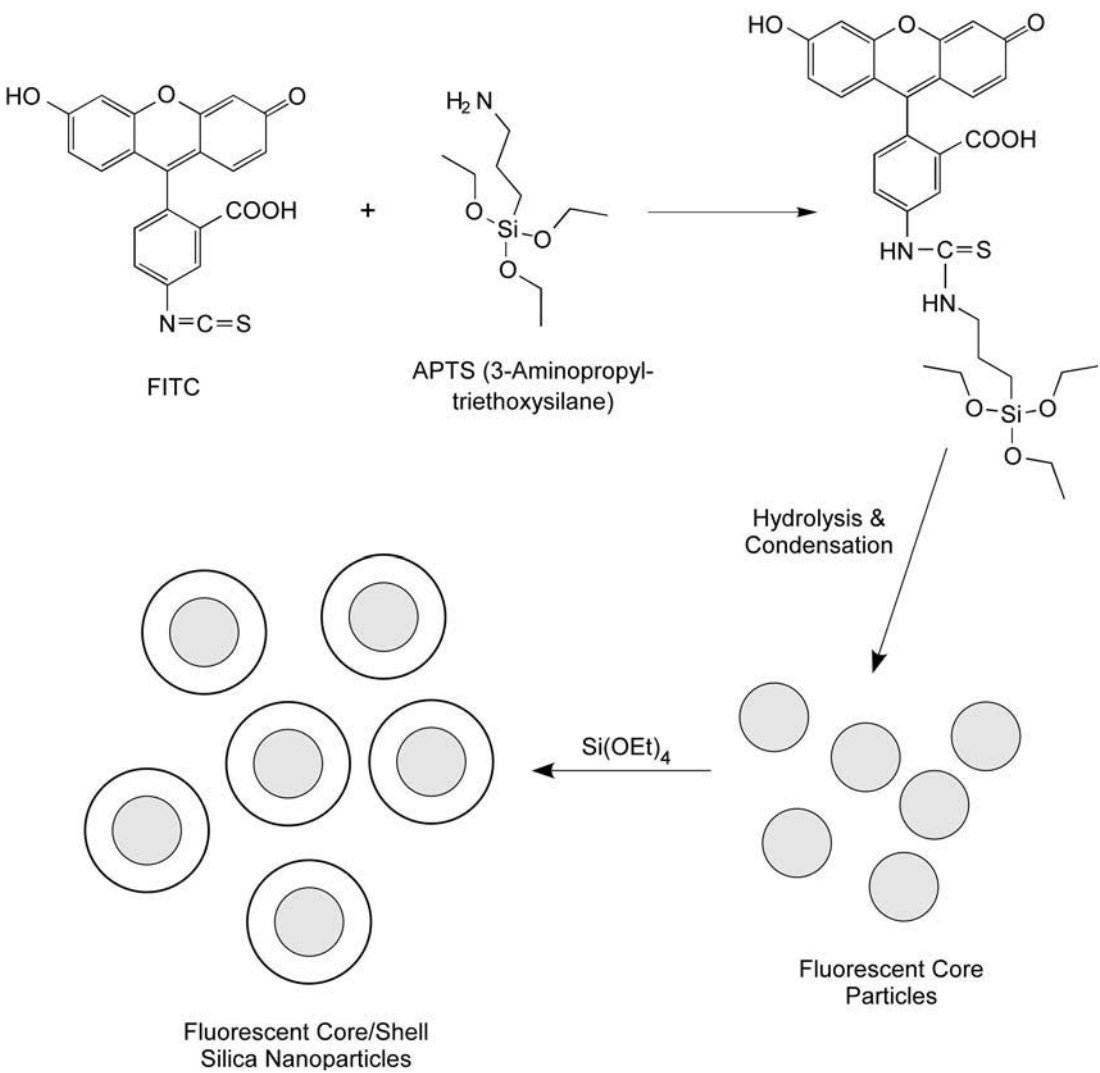


Figure 14.25 The preparation of highly controlled fluorescent silica nanoparticles can be done by first polymerizing APTS that has been covalently modified with an amine-reactive dye to form fluorescent core particles. The core then is capped by a shell of silica by polymerization of TEOS. The shell layer can be further derivatized with silane coupling agents to provide functional groups for conjugation.

The method developed by Ow *et al.* permits control over the size of the particles and allows the incorporation of virtually any organic dye into silica, provided it can be first conjugated to a silane derivative to form the core. Fluorescent particles made by this procedure may be made as monodisperse populations having diameters from less than 10 nm to over 1 μm sized spheres, with a high degree of precision. The core size also can be varied by changing the concentration of the dye-silane derivative during the condensation process. The procedure for making these particles is similar to that described above using the method of van Blaaderen and Vrij (1992), but without the addition of TEOS for creation of the dye-silane core. Exact procedures are given in Wiesner *et al.* (2006).

In the preparation of 15 nm core–shell fluorescent silica particles, Ow *et al.* (2004) reported that the naked core (2.2 nm) alone produced a fluorescence intensity of less than the free dye in solution, presumably due to dye quenching. However, upon addition of the outer silica shell around the core, the brightness of the particles increased to 30 times that of the free dye (using tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)). They speculate that shell may protect the core from solvent effects, as evidenced by a lack of spectral shift upon changing the solvent in which the particles are suspended.

The enhanced photophysical properties of these fluorescent core–shell silica nanoparticles make them potentially as useful as semiconductor quantum dots for bioconjugation purposes. In this case, standard, commercially available, organic dyes can be incorporated into the silica nanoparticles to provide a range of emission properties as diverse as those available using the dyes alone. In addition, once encapsulated in the particles, the dyes display much better photostability and increased fluorescence (brightness) compared to the free dyes in solution. A major advantage of silica-based particles is that they are known to have greater biocompatibility than quantum dots in that they are nontoxic, hydrophilic, and can be conjugated to proteins and other targeting molecules with relative ease. The ability to add any desired fluorescence characteristic to such particles simply by choosing the appropriate organic dye or metal chelate luminescent molecule makes dye-doped silica nanoparticles especially useful for bioapplications.

5.2. Silane Functionalization of Silica Particles

Surface functionalization of silica particles or fluorescent silica particles typically is done using functional alkyl silanes. The process may be used to add a reactive group to the surface of the particles for spontaneous coupling to biomolecules or it may be used to add the appropriate nucleophilic group to the surface, such as an amine or a carboxylate. Silane modification chemistry is discussed in more detail in Chapter 13.

The following protocol for modification of silica nanoparticles is based on the method of Zhao *et al.* (2004), which describes the addition of amine functionalities using trimethoxysilylpropyldiethylenetriamine. Other functional silane modifications may be done similarly.

Protocol

1. Add 32 mg of silica nanoparticles (fluorescent or plain) to 20 ml of 1 mM acetic acid containing 1 percent trimethoxysilyl-propyldiethylenetriamine with stirring. Other concentrations of silane derivatives used for particle modification typically range from 1 to 5

percent (w/w). Optimization of this concentration may have to be done for a particular silica particle size and type.
2. React with mixing for 30 minutes at room temperature.
3. Wash the amine-derivatized particles at least 3 times with water using centrifugation to remove excess reactants.
4. Store the particles in a suitable buffer at neutral or slightly acidic pH containing a preservative.

The amine-modified silica particles may be used to couple with carboxylate-containing ligands using a carbodiimide reaction. Similar coupling protocols may be used as that previously described for amine-containing polymer particles (Section 4, this chapter). The amine-particles also may be further derivatized by reaction with succinic anhydride to create carboxylated particles for coupling to proteins or other amine-containing ligands.

5. To prepare the succinylated carboxylate derivative of the amine particles, wash the particles with water and then into DMF.
6. Suspend the amine-particles in DMF containing 10 percent succinic anhydride.
7. React for 6 hours under nitrogen gas with mixing.
8. Wash the carboxylated particles at least 3 times with DMF by centrifugation. Resuspend in water and wash 3 times with water to remove DMF. Store the particles in water or a suitable buffer at neutral or slightly acidic pH.

Carboxylated silica particles may be coupled with amine-containing ligands, such as proteins, using a carbodiimide reaction with EDC. A similar protocol to that previously described for coupling to carboxylate polymer particles may be used. The following protocol is based on the method of Zhao *et al.* (2004), which was used for immobilizing monoclonal antibodies to *E. coli* O157.

Protocol

1. Suspend the carboxylated silica particles from step 8, above, in 10 ml of 0.1 M MES, pH 6.8.
2. With mixing, add 500 mg of EDC and 500 mg of NHS (or sulfo-NHS).
3. React for 25 minutes at room temperature with stirring.
4. Quickly wash the activated particles with water using centrifugation to remove excess reactants. Resuspend the washed particles in 10 ml of 0.1 M sodium phosphate, pH 7.3.
5. Add a quantity of protein or antibody to the activated particles representing a 1–10 × excess of protein over the calculated monolayer for the type of particles used. For coupling a limiting amount of antibody to the activated fluorescent particles, something that may be desirable when using expensive monoclonals, the reaction should be carried out in very dilute particle suspension (i.e., 0.1 mg/ml) to prevent aggregation of particles due to the possibility of one antibody reacting with more than one particle. Zhao *et al.* (2004) used an antibody concentration of just 5 μg/ml to create successfully anti-bacterial fluorescent particles.
6. React for 2–4 hours at room temperature.
7. Add a blocking agent, such as a non-relevant protein (e.g., BSA) to a final concentration of 1 percent to mask any nonspecific binding sites and to couple with any remaining reactive groups on the silica particle surface. This is important especially if a limiting amount of antibody was initially reacted with the particles in step 5. React for 30 minutes to 1 hour at room temperature.
8. Wash the particles several times with PBS, pH 7.2, to remove excess protein and reaction by-products. Store products in neutral or slightly acidic buffer containing a preservative.

15

Buckyballs, Fullerenes, and Carbon Nanotubes

1. Buckyballs and Fullerenes

1.1. Properties of Fullerenes

Carbon is an incredible element that is able to form structures having highly diverse properties depending on its bonding patterns and three-dimensional organization. Natural allotropes of carbon include diamond, graphite, amorphous carbon, and several other known forms (Figure 15.1). Depending on the bond structure and atomic orientation that carbon takes on within the structure of an allotrope, the resultant characteristics can range from the hardest known abrasive mineral, diamond, to the extremely soft, graphite, which is used as a lubricant.

In 1985, the story of carbon allotropes took a dramatic turn with the discovery of C_{60}, which resulted in a new type of carbon structure, called the fullerenes (Kroto *et al.*, 1985). This discovery earned the 1996 Nobel Prize in chemistry for Harold Kroto, Robert Curl, and

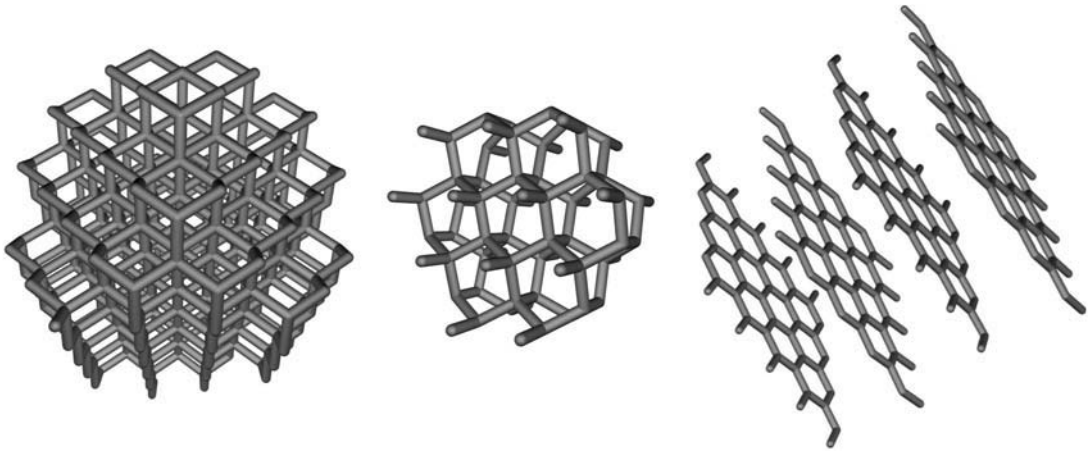


Figure 15.1 Three major allotropes of carbon (l to r): diamond, lonsdaleite, and graphite.

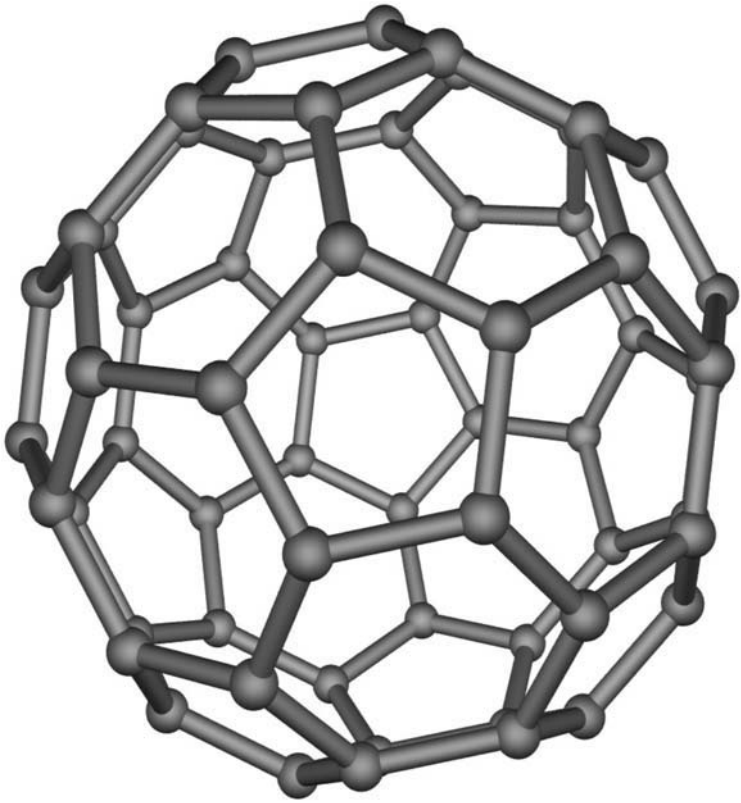


Figure 15.2 The structure of a C_{60} fullerene, also called a Buckyball.

Richard Smalley. Buckminsterfullerene (named after Buckminster Fuller for his geodesic dome architectural design) is a spherical cage of carbon having 60 atoms forming a truncated icosahedron of average diameter 0.72 nm, which contains 12 pentagons and 20 hexagons of bonded carbon (Figure 15.2). The shape is exactly the same as a modern soccer ball, with the pentagon and hexagon configurations clearly outlined on its surface.

There now are known to be a whole family of caged carbon structures having various numbers of carbon atoms, including C_{30}, C_{50}, C_{70}, C_{72}, C_{76}, C_{84}, and the huge C_{540}. The name "fullerene" has replaced the unwieldy, "Buckminsterfullerene" used to describe this general spheroid structure of carbon, although they still are referred to as "Buckyballs".

Of all the fullerene forms, the nearly spherical properties of C_{60} have attracted the greatest attention, especially in the field of bioconjugation. In addition to its physical properties, C_{60} fullerenes have unique photo-optical and electro-chemical properties, which make them useful as carriers for biomedical research applications. For instance, upon exposure to light C_{60} will generate singlet oxygen, which can be used *in vivo* to cleave biological molecules, particularly DNA and RNA. Studies indicate that irradiation of C_{60} in solution can be used to destroy virus contamination (Kasermann and Kempf, 1997). Solutions of Buckminsterfullerene are a deep purple color, whereas other sizes of fullerenes display a variety of other colors.

Fullerene C_{60} also functions efficiently as an antioxidant, actually being better than other lipid-soluble antioxidants at scavenging reactive oxygen species (ROS) (Wang *et al.*, 1999). Water-soluble derivatives of C_{60}, such as a poly-hydroxyl form, are able to function in the same respect in aqueous environments.

Pure fullerenes are insoluble in aqueous environments and only sparingly soluble in many organic solvents. The greatest solubility is found in 1,2,4-trichlorobenzene (20 mg/ml), carbon disulfide (12 mg/ml), toluene (3.2 mg/ml), and benzene (1.8 mg/ml) (Wikipedia.org). Solubility calculations have been performed on C_{60} in 75 different organic solvents (Sivaraman *et al.*, 2001).

1.2. Modification of Fullerenes

Many chemical derivatization methods have been developed to afford fullerene solubility in particular environments and to provide functional handles for bioconjugation (Bosi *et al.*, 2003). The combination of adding polar groups and reactive functionalities to fullerenes, such as —COOH, —NH_2, and —OH groups, provides water solubility and bioconjugation targets. Examples of these modifications include the method of Brettreich and Hirsch (1998) to add multiple carboxylates in a dendritic fashion and Wang *et al.* (1999) who added multiple pairs of carboxylates to the surface carbons. In addition, Cusan *et al.* (2002) developed a C_{60}–PEG dendrimer-based diamine derivative using a substituted fulleropyrrolidine modification linked to the surface. Polymer carriers also have been used to provide water solubility and sites of attachment. Cyclodextrins have been found to be excellent carriers of C_{60} by holding the fullerene within its hydrophobic core (Andersson *et al.*, 1992; Braun, 1997; Samal and Geckeler, 2000; Filippone *et al.*, 2002).

In many methods for derivatization of C_{60}, the initial modification is based on the reaction at a 6,6 ring junction on the fullerene with an azomethine ylide to form the 1,3-dipolar cycloaddition product, a fulleropyrrolidine (Prato *et al.*, 1996). The reaction is done overnight with heating to reflux in organic solvent. Typical reactants that combine with C_{60} in this reaction include an *N*-glycine derivative (with a constituent off the α-amino group) and an aldehyde derivative, which gives the fulleropyrrolidine compound according to Figure 15.3. By judicious choice of the right starting materials, the process provides a range of derivative possibilities to employ fullerenes in various bioconjugate applications. If the reactants are added in large excess over the concentration of the fullerene, then up to 9 such pyrrolidine groups can be introduced per C_{60} molecule. The number of modifications actually ends up being a bell shaped curve from 5 to 9 pyrrolidine derivatives, with a peak at 7 modifications (Prato and Maggini, 1998). By controlling the length of the reaction, a mono-substituted product can be obtained in 40–50 percent yields.

In addition, the use of appropriate hydrophilic constituents on the aldehyde or glycine reactants can result in excellent water solubility of the C_{60} derivative. Two such modification arms can be added simultaneously to the pyrrolidine ring, thus providing a functional group for further conjugation and a hydrophilic arm for increased water solubility. PEG derivatives have been formed in this manner, which create highly soluble fullerene derivatives.

The following procedure adapted from Prato *et al.* (1996) is an example of how glycine and formaldehyde derivatives may be used to create fullerene modifications for subsequent bioconjugation purposes.

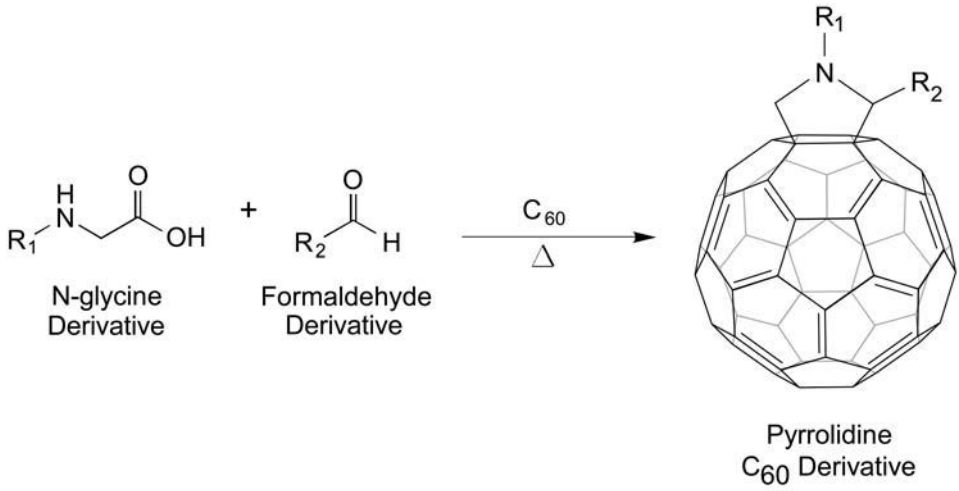


Figure 15.3 The reaction of a glycine derivative and a formaldehyde compound with a C_{60} molecule leads to the formation of a fulleropyrrolidine.

Protocol

1. In a fume hood, prepare a solution of 100 mg of C_{60} dissolved in 100 ml of toluene.
2. Add to the fullerene solution with mixing, 25 mg of *N*-methyl glycine (sarcosine) and 20 mg of paraformaldehyde.
3. React by refluxing for 2 hours with mixing.
4. Remove solvent under vacuum and purify the *N*-methyl-3,4-fulleropyrrolidine (Figure 15.4) by flash chromatography using toluene as eluent.

A similar preparative procedure can be used to form the *N*-(triphenylmethyl)-3,4-fulleropyrrolidine, which is a trityl-protected amine derivative that may be deprotected using trifluoromethanesulfonic acid (TFMSA) to create a free secondary amine for conjugation. Kurz *et al.* (1998) used this C_{60} derivative to react with 3-maleimidopropionyl chloride to form a sulfhydryl-reactive fullerene. Although this derivative was not soluble in aqueous buffers, it was reactive enough as a micro-precipitate to be conjugated to thiol-containing proteins using the following procedure.

Protocol

1. The thiol-containing protein is dissolved as a 60 μM solution in 20 mM HEPES, pH 7.
2. Add the maleimide-C_{60} derivative to the protein solution at 100× mole excess with stirring. The addition of detergent to the solution may increase the solubility of the fullerene compound.
3. React with mixing at 4°C for 72 hours.
4. Purify the labeled protein by size exclusion chromatography using a column with an exclusion limit of molecular weight 5,000.

The nitrogen group in fulleropyrrolidines can be used for conjugation with crosslinking agents or hydrophilic biotinylation compounds for subsequent use in bioconjugation reactions.

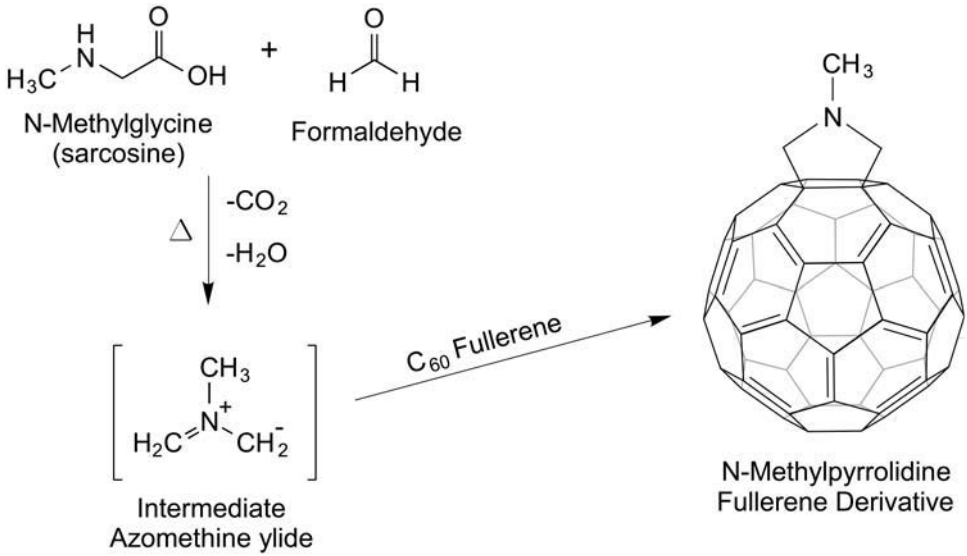


Figure 15.4 The synthesis of *N*-methylpyrrolidine-C_{60} proceeds through an intermediate azomethine ylide by the reaction of *N*-methylglycine plus formaldehyde upon heating.

To create the free secondary amine group (N-H) fulleropyrrolidine, an amine-protected starting material can be used in the reaction (Cai *et al*., 2006). For instance, a trityl-oxazolidinone (using either triphenylmethyl- or better, 4-methoxytriphenylmethyl-protecting groups) can be reacted with C_{60} to yield the trityl-protected pyrrolidine (Figure 15.5).

The following procedure for creating the N-H fulleropyrrolidine is adapted from Maggini *et al*. (1994), Prato and Maggini (1998), and Prato *et al*. (1996). Extreme care should be taken when using TFMSA, as this acid is 30 times stronger than concentrated sulfuric acid.

Protocol

1. Dissolve 100 mg of C_{60} in 130 ml of chlorobenzene in a fume hood.
2. Add with mixing to the fullerene solution 54 mg of either *N*-(triphenylmethyl)-5-oxazolidinone or the same amount of the methoxytrityl oxazolidinone.
3. Reflux overnight in a fume hood with constant mixing.
4. Remove the solvent under vacuum and purify the residue using flash chromatography with an 8:2 mixture of petroleum ether/toluene as the eluent.
5. Remove the trityl-protecting group by dissolving the derivatized fulleropyrrolidine in 5 ml of dichloromethane in a fume hood and then adding 50 µl of TFMSA (caution!) with stirring.
6. React at room temperature for 1 hour to remove the trityl-protecting group, yielding the N-H pyrrolidine derivative of C_{60}.

Capaccio *et al*. (2005) used the N-H fulleropyrrolidine derivative to couple long-chain biotin to the C_{60} using the carbodiimide dicyclohexyl carbodiimide (DCC) in a pyridine/DMF/CH_2Cl_2 solvent mixture and reacting overnight at room temperature. This derivative was prepared using a hydrophobic, aliphatic long-chain biotin, but similar derivatives could be prepared

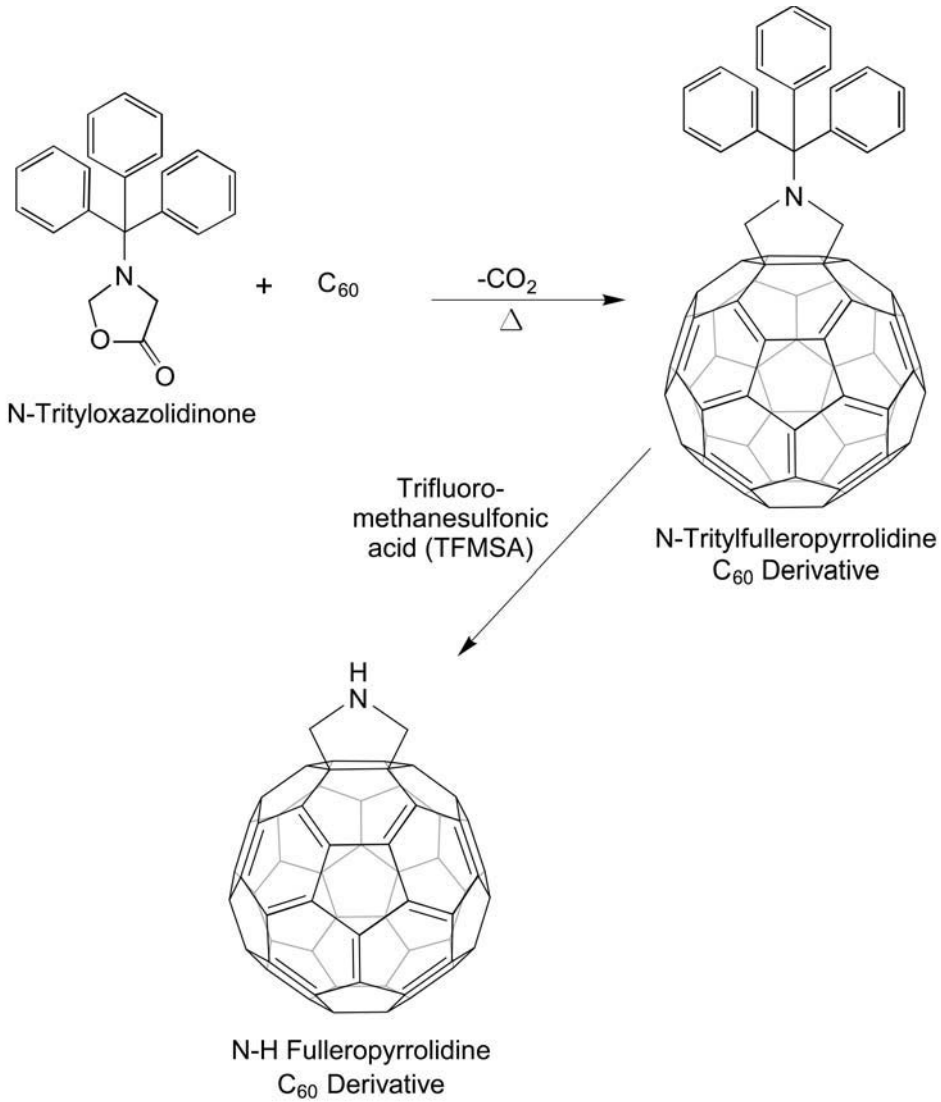


Figure 15.5 A trityl-protected pyrrolidine derivative of C_{60} can be prepared by the reaction of *N*-trityl-oxazolidinone with a fullerene. Deprotection of the trityl group using methanesulfonic acid gives the secondary amine, which can be used in further conjugation reactions.

using a biotin-PEG-carboxylate compound (e.g., NHS–PEG_4–biotin) to create a hydrophilic fullerene modification (Figure 15.6). The biotinylated fullerene could be used with streptavidin and another biotinylated enzyme to create a bioconjugate.

Similar to the fullerene modifications using either glycine/formaldehyde derivatives or oxazolidinone compounds, Maggini and Scorrano (1993) found that aziridines could yield similar pyrrolidine derivatives. Heating aziridine compounds in toluene was found to result in ring

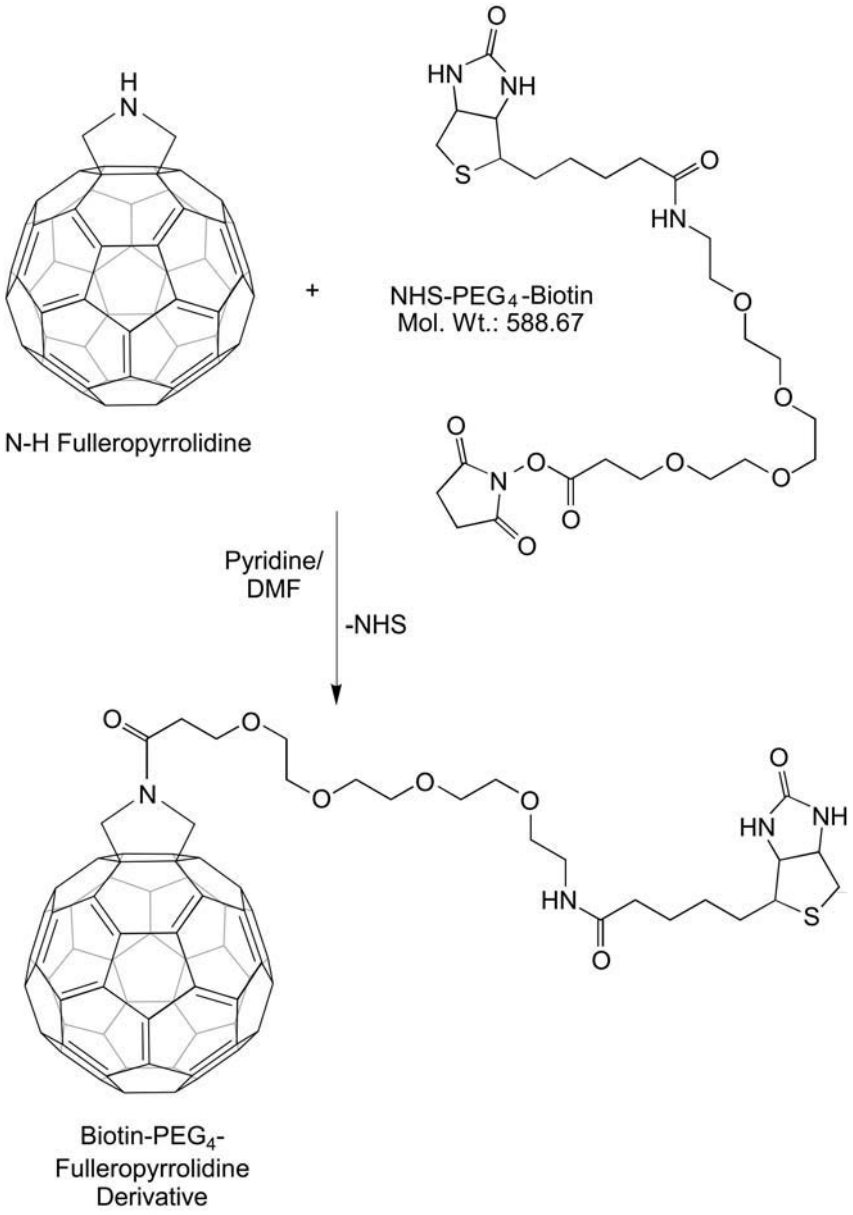


Figure 15.6 Reaction of an N-H fulleropyrrolidine with NHS–PEG_4–biotin creates the biotinylated C_{60} derivative via an amide bond.

opening of the aziridine with subsequent covalent linking to the C_{60} ring system (Figure 15.7). Thus, synthesizing pyrrolidine derivatives of fullerenes can be done by several routes with success.

Another route to the preparation of C_{60} derivatives involves the reaction of a halogen derivative of diethylmalonate in the presence of a strong base with fullerenes, which results in a

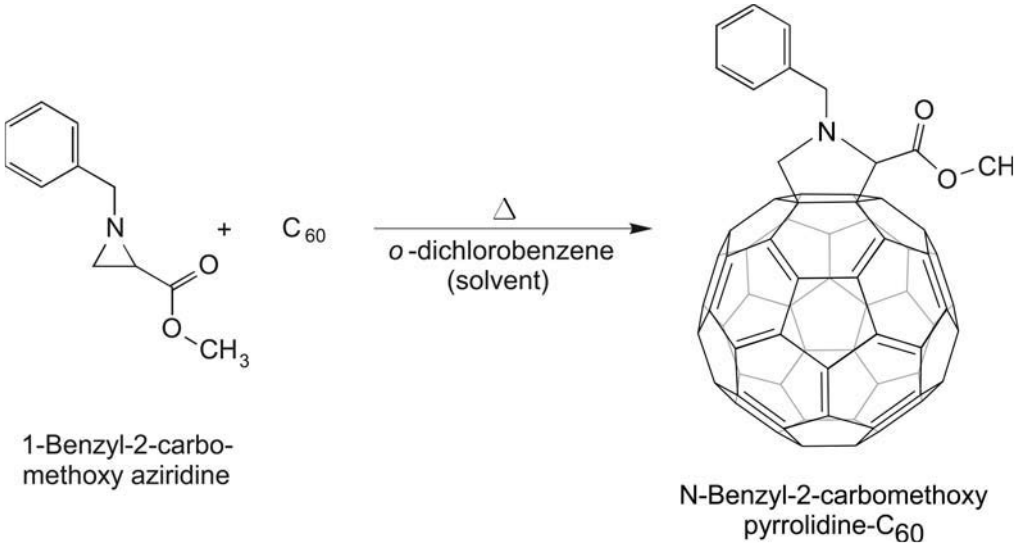


Figure 15.7 The reaction of aziridine derivatives with fullerenes also can give pyrrolidine derivatives useful for bioconjugation.

cyclopropanation product (Bingel, 1993; Isaacs and Diederich, 1993; Isaacs *et al*., 1993). Using this reaction, various malonate derivatives of C_{60} can be made to facilitate production of bioconjugates (Zakharian *et al*., 2005). This reaction often is called the Bingel reaction (or Bingel–Hirsch addition) after the publication by the author in 1993 (see also Hirsch *et al*., 1994). The reaction can yield carboxylate derivatives that then can be used to couple biomolecules through carbodiimide conjugation (Figure 15.8).

The following protocol represents a C_{60} modification with an ethylmalonate derivative using the Bingel-type reaction, which creates an amine functional group on the fullerene surface, and is based on the method of Zakharian *et al*., 2005 (Figure 15.9). Ashcroft *et al*. (2006) used this method to create C_{60} immunoconjugates with the murine anti-gp240 melanoma antibody, which was made water-soluble through the additional modification of the fullerene with hydrophilic malonodiserinolamide groups.

Protocol

1. In a fume hood, prepare a solution of 1.16 g *tert*-butyl-*N*-(3-hydroxypropyl)carbamate (Aldrich) in 100 ml of dry CH_2Cl_2 and add 1 ml of pyridine with stirring.
2. Cool the solution to 0°C and slowly add 1 g of ethylmalonyl chloride (Aldrich) under nitrogen with stirring.
3. React for 12 hours with stirring at room temperature.
4. The resultant ethylmalonate-protected-amine compound is concentrated *in vacuo* and then purified using silica gel chromatography with a hexane/ethyl acetate (1:1) eluent.
5. In a fume hood, dissolve 400 mg of C_{60} in 700 ml of toluene with stirring.
6. Add to the C_{60} solution 100 mg of the purified ethylmalonate-protected-amine compound from step 4 along with 88 mg of I_2 and 105 mg of DBU (Aldrich).

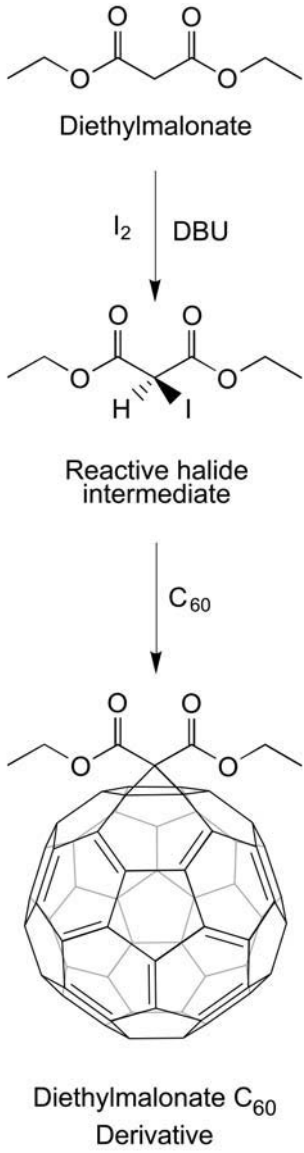


Figure 15.8 The Bingel reaction for the modification of fullerenes involves the *in situ* formation of a reactive halogen species in the presence of the strong base DBU. The cyclopropanation product can be used to create many bioconjugates.

7. React for 30 minutes at room temperature with mixing.
8. Remove the solvent *in vacuo* and purify the resultant mixture using silica gel chromatography. The initial eluent is toluene, which will remove the unreacted C_{60}, and then followed by a 10:1 mixture of toluene/ethyl acetate to elute the desired C_{60}-protected-amine derivative.

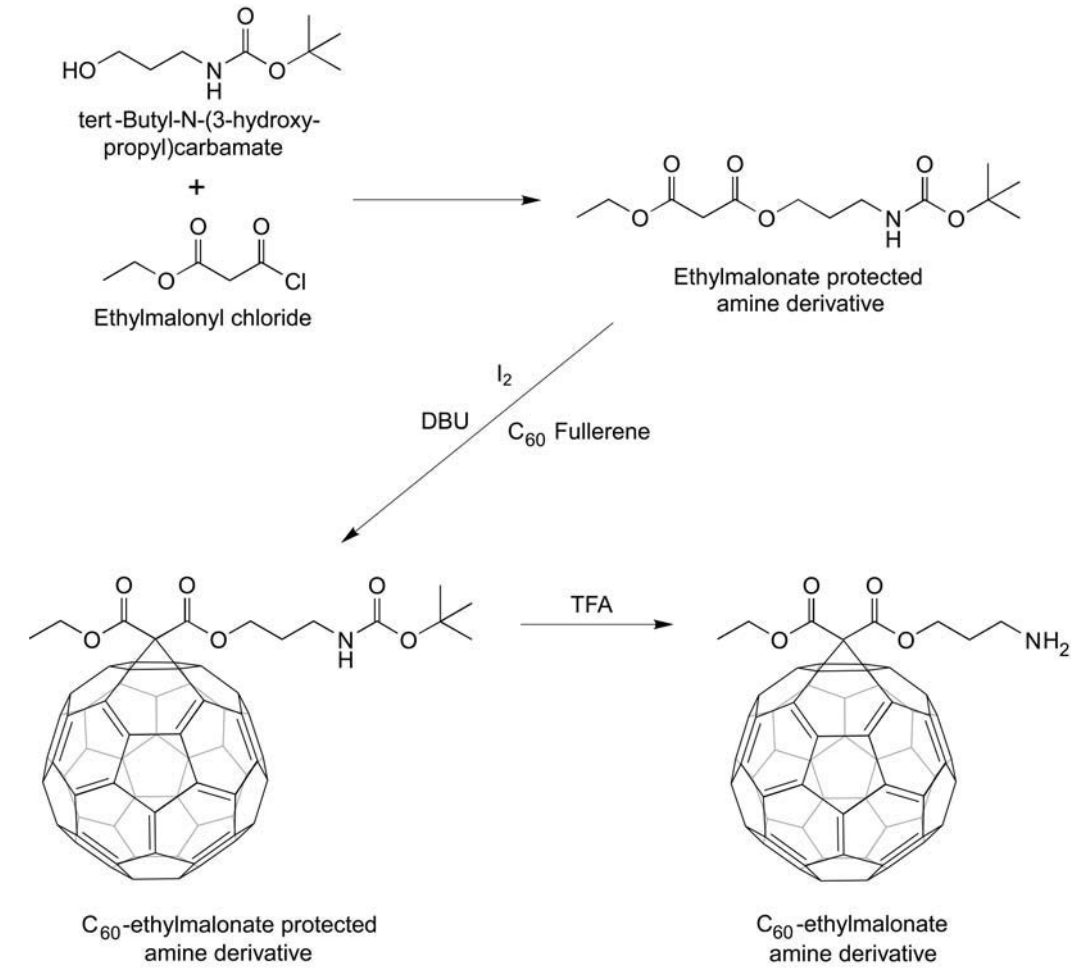


Figure 15.9 The reaction of the amine-blocked derivative of 3-hydroxypropylamine with ethylmalonyl chloride gives an ethylmalonate-protected-amine compound, which can be used in the Bingel reaction to create an amine group on a fullerene surface. Reaction with C_{60} in the presence of I_2 and DBU gives the cyclopropanation product that can be deprotected with TFA to yield the free amine.

9. To remove the *tert*-butyl-protecting group on the amine, in a fume hood add 190 mg of the C_{60}-protected-amine derivative from step 8 to 50 ml CH_2Cl_2 with stirring. Add to this solution with stirring 50 ml of TFA and mix for 30 minutes. Remove the solvent *in vacuo* to yield the final amine–C_{60} compound.

The amine group on the C_{60} fullerene may be used to couple carboxylate-containing spacers or affinity ligands using a carbodiimide conjugation reaction to form an amide linkage. The reaction may be done in organic solvent or aqueous buffer, depending on the hydrophilicity of the fullerene derivative. A mixture of organic and aqueous solution also may be done if the ligand is more soluble in an aqueous environment.

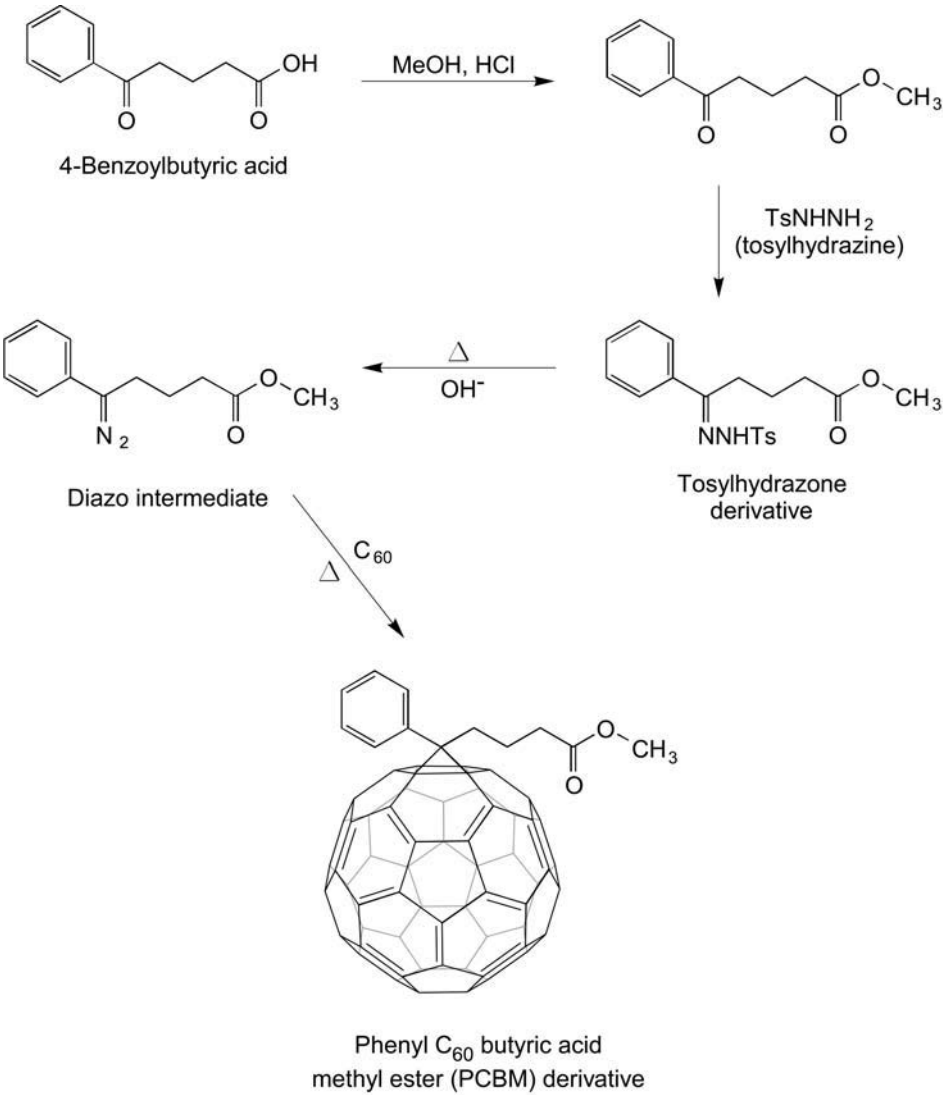


Figure 15.10 Fullerene-PCBM derivatives can be prepared using reactive diazo intermediates, which yield a cyclopropanation product similar to the Bingel reaction derivatives.

A similar fullerene modification process developed by Hummelen *et al.* (1995) resulted in a number of phenyl C_{60} butyric acid methyl ester (PCBM) derivatives, which now are commercially available (Nano-C, Solenne). The reaction proceeds through the creation of a diazo derivative of methyl 4-benzoylbutyrate, which then is reacted with the aromatic ring system of C_{60} to give the cycloaddition product (Figure 15.10). The conjugate forms through the formation of both a 5,6 ring addition product (fulleroid) or a 6,6 ring addition product (methanofullerene). The fulleroid product results in breaking the 5,6 ring junction on the C_{60} molecule with dimeric bond formation to the aryl methyl group on PCBM.

The PCBM methyl ester can be used for coupling amine-containing ligands after removal of the methyl group and activation of the carboxylate using a number of different reaction strategies. Hummelen *et al.* (1995) successfully coupled cholestanol and histamine to the fullerene-PCBM derivative (after acid chloride formation) for use in fabrication of photodetectors and biological studies, respectively. For specific applications of PCBM-fullerenes, see Shaheen *et al.* (2001), Brabec *et al.* (2001), Yu *et al.* (1995), Mecher *et al.* (2002), Meijer *et al.* (2003), van Duren *et al.* (2004), and Anthopoulos *et al.* (2004).

Various commercial suppliers now offer fullerene derivatives with functionalities available for bioconjugation, including carboxylic and poly-hydroxylic derivatives, which are very hydrophilic and water-soluble (BuckyUSA, NanoLab, NanoNB, Nano-C, and Aldrich).

2. Carbon Nanotubes

2.1. Nanotube Properties

A cousin of the spheroidal fullerene molecules is carbon nanotubes. These are cylindrical fullerenes with either open or closed ends and consist of carbon atoms arranged in a hexagonal pattern. There are two main families of carbon nanotubes that are distinguished by being either single-walled nanotubes (SWNTs) or multi-walled nanotubes (MWNTs). SWNTs actually are a graphene sheet that is seamlessly wound into a cylinder. MWNTs consist of multiple SWNTs that are concentrically wound around each other and nested together to create a tubes-within-tubes configuration (Figure 15.11). The type of nanotube also is determined by manner in which the hexagonal pattern of carbon rings is arranged in the cylindrical structure.

In most publications, Iijima is given credit for the discovery in 1991 of the nanotube structure of carbon (Iijima, 1991; Bethune *et al.*, 1993; Iijima and Ichihashi, 1993). However, it has been said that Oberlin *et al.* (1976) also imaged carbon nanotubes, perhaps even SWNTs. Incredibly, nearly a century earlier, there was a study on the thermal decomposition of methane that resulted in the formation of long carbon strands, which were proposed at the time as a candidate for filaments in light bulbs (see Bacon and Bowman, 1957).

SWNTs are typically only a few nanometers in diameter (0.4 to ~3 nm), but MWNTs can be from about 1.4 nm to over 100 nm in diameter, depending on the number of concentric nanotubes making up the bundle (Baughman *et al.*, 2002). However, carbon nanotubes can be from nanometers to millimeters or even microns in length, depending on how they are made. The length-to-diameter ratio typically exceeds 10,000 in most preparations. This unique molecular structure results in fascinating properties, which include extremely high tensile strength, electrical conductivity (or even semiconductor properties, depending on how the graphene sheet is wrapped), resistance to heat, and a great deal of chemical robustness. Nanotubes are being explored for use in applications ranging from electronics, optics, material science, biomedicine, biosensors, hydrogen storage, and nanoelectromechanical systems (NEMS) fabrication. The rate of growth in nanotube related patents and publications have been nearly on an exponential increase since the early 1990s, demonstrating the broad applications they can be used in (Baughman *et al.*, 2002; Park *et al.*, 2003).

The tensile strength of carbon nanotubes has been determined to be over 50 times that of high-carbon steel (Yu *et al.*, 2000). The strength of the bond structure in carbon nanotubes

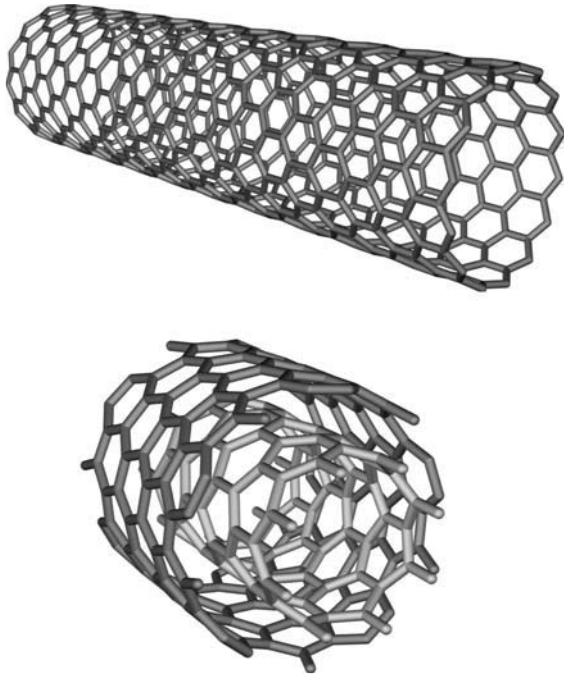


Figure 15.11 An example of a single-walled carbon nanotube (a) and a multi-walled carbon nanotube (b). Multi-walled varieties can consist of numerous tubes within tubes.

results from the fact that they are entirely sp^2 bonds, which are even stronger than the sp^3 carbon bonds in diamond. The addition of nanotubes to polymers, metals, and other structural materials has been used to add considerable strength to these materials. In fact, a carbon nanotube tether into low Earth orbit has been proposed as the only way of creating an elevator system into space.

The orbital bonding nature within carbon nanotubes creates unique electrical properties within a non-metallic molecule, which is a result of the delocalization of the π-electron donated by each atom. Electrical conductivity can take place along the entire nanotube due to the freedom of π-electron flow, making possible the design of circuits of extremely low nanometer diameter.

Synthetic methods for the production of carbon nanotubes include arc discharge from graphite electrodes (Iijima, 1991; Collins and Avouris, 2000), pulsed laser ablation of a graphite substrate under high temperature (Guo *et al.*, 1995a, b), chemical vapor deposition (Jose-Yacaman *et al.*, 1993; Ren *et al.*, 1998), and high-pressure carbon monoxide (HiPco) method developed by Carbon Nanotechnologies, Inc. Methods for nanotube purification have been developed that result in 99.9 percent pure single-wall nanotubes (Chiang *et al.*, 2001).

Unlike the smaller, spheroidal fullerenes discussed previously, carbon nanotubes are not easily solubilized, even in organic solution. The reality is that all SWNTs and MWNTs are insoluble in all solvent systems. They also have a strong tendency to bind together and aggregate due to van der Waals attractive forces along the length of the nanotube. Since the length-to-diameter

ratio is so high for nanotubes, bundles often are observed to be knotted masses, which are very difficult to unravel. The best that can be done is to disrupt the bundle and form a dispersion of the nanotubes in a solvent medium.

Some of the better solvents for pure SWNTs are the amide-containing ones, like DMF or *N*-methylpyrrolidone, but they still do not permit full dissolution, just dispersion (Boul *et al*., 1999; Liu *et al*., 1999). The addition of surfactants to carbon nanotube suspensions can aid in their solubilization, and even permit their complete dispersion in aqueous solution. The hydrophobic tails of surfactant molecules adsorb onto the surface of the carbon nanotube, while the hydrophilic parts permit interaction with the surrounding polar solvent medium.

2.2. Nanotube Functionalization

An important route to solubilization of carbon nanotubes is to functionalize their surface to form groups that are more soluble in the desired solvent environment. It has been shown that acid treatment of nanotube bundles, particularly with HCl or HNO_3 at elevated temperatures, opens up the aggregate structure, reduces nanotube length, and facilitates dispersion (An *et al*., 2004; Kordás *et al*., 2006). Nitric acid treatment oxidizes the nanotubes at the defect sites of the outer graphene sheet, especially at the open ends (Hirsch, 2002; Álvaro *et al*., 2004), and creates carbonyl, carboxyl, and hydroxyl groups, which aid in their solubility in polar solvents.

Such carbonyls may be further oxidized using potassium permanganate ($KMnO_4$) and perchloric acid ($HClO_4$) to convert all of these groups into carboxylic acids. Once functionalized in this manner, the nanotubes can be fully dispersed in aqueous systems. Kordás *et al*. (2006) used these derivatives to print nanotube patterns on paper or polymer surfaces to create conductive patterns for potential use in electronic circuitry. The carboxylates also may be used as conjugation sites to link other ligands or proteins to the nanotube surface using a carbodiimide reaction as previously discussed (Section 1, this chapter; Chapter 2, Section 1.11; Chapter 3, Section 1).

Untreated carbon nanotubes nonspecifically adsorb protein in an irreversible manner much like the noncovalent adsorption of protein onto hydrophobic surfaces (Chen *et al*., 2003). It is essential, therefore, to modify the surfaces of nanotubes to prevent hydrophobic binding of biomolecules, especially if the resultant conjugates are to be used in biological assays or systems. Two main strategies have been used to make nanotubes biocompatible: (1) the noncovalent modification of the graphene surface with amphipathic molecules, which have functional groups that can be used for conjugation or (2) the covalent modification of the outer nanotube surface to promote hydrophilicity and create functional groups for coupling other molecules.

2.3. Detergent or Lipid Modification of Carbon Nanotubes

Detergents have been used for simple solubilization of SWNTs in aqueous solution. Ionic detergents such as SDS will coat the nanotube surface and expose the negatively charged sulfonate groups to the surrounding aqueous environment, thus allowing SWNT dispersion in aqueous

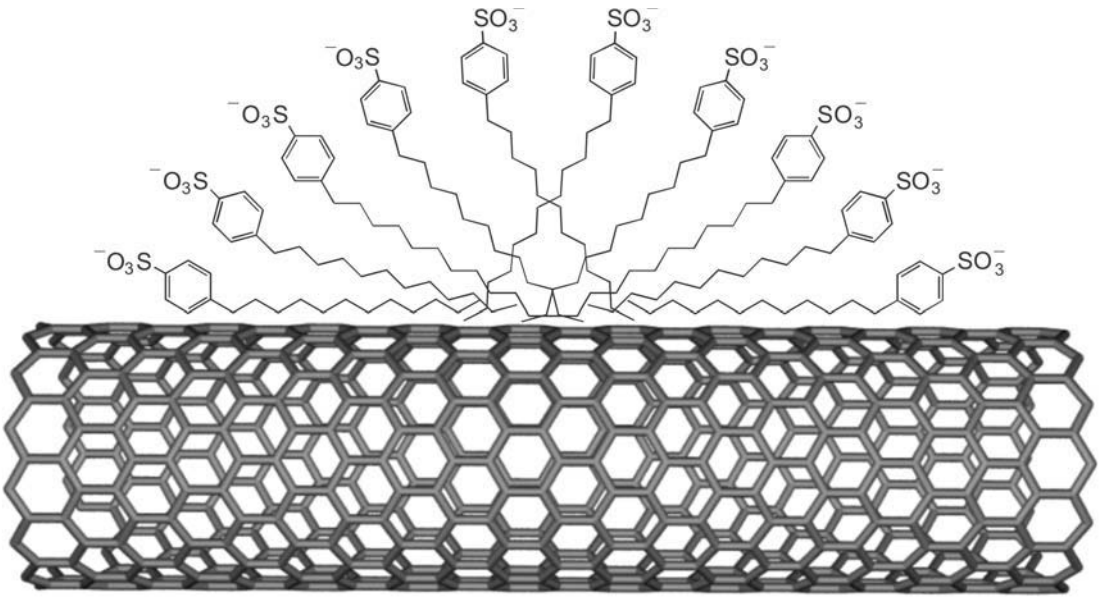


Figure 15.12 Detergent molecules can be used to solubilize carbon nanotubes by adsorption onto the surface through hydrophobic interactions and create half-micelle structures with the hydrophilic head groups facing outward into the aqueous environment.

environments. Similarly, nonionic detergents such as Triton X-100 will coat the tubes and present their hydrophilic groups to the aqueous phase. Fischer *et al.* found that coating SWNTs with sodium dodecylbenzene sulfonate resulted in the best solubilization properties with long-term stability of the nanotubes in aqueous buffers (Zhou *et al.*, 2004). The detergent molecules don't merely lie in a random pattern on the nanotube surface; they coat the SWNT in a series of half-micelle structures, which create small knobs along its length (Figure 15.12).

Detergents also have been exploited in the noncovalent modification of carbon nanotubes by using modified detergents containing a coupled affinity ligand, which is linked to the hydrophilic part of the detergent molecule. Detergents that contain both a hydrophobic portion and a hydrophilic part with at least one terminal functional group for conjugation can be used in this process. The hydrophobic tail of many detergents will strongly adsorb to the nanotube's outer graphene cylinder leaving the hydrophilic portions pointing outward and available for conjugation, if they contain an appropriate functional group. The result is a hydrophilic surface that is completely masked to prevent nonspecific protein binding. This approach to nanotube functionalization also leaves the chemical structure of the graphene cylinder unaffected, thus avoiding defects that could alter its electronic properties.

As an example of this strategy, Chen *et al.* (2003) used an activated Tween 20 detergent to coat SWNTs for subsequent conjugation to biotin, protein A, and U1A antigen. Tween 20 is a poly-oxyethylene sorbitan monolaurate compound with 20 ethylene oxide units, 1 sorbitol unit, and 1 lauric acid group esterified as the hydrophobic tail. The fatty acid group avidly adsorbs to the carbon nanotube surface, leaving the three PEG arms, each containing terminal

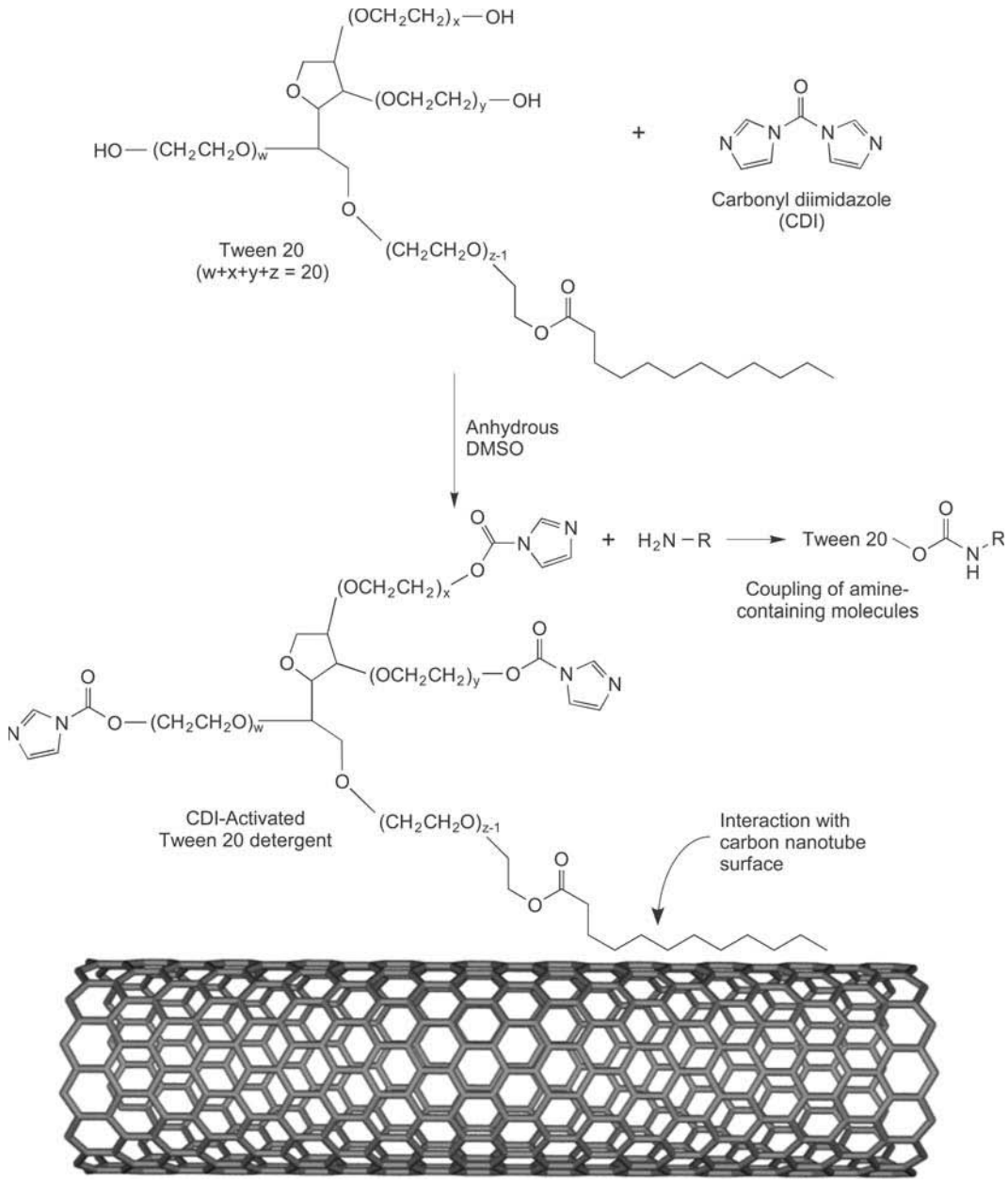


Figure 15.13 Tween 20 can be activated with CDI using its hydroxyl groups to create an amine-reactive imidazole carbamate intermediate that then can be used to coat a carbon nanotube. The result is an activated nanotube that can be used to couple proteins and other amine-containing molecules.

hydroxyl groups, sticking out from the coating to create an extremely biocompatible construct (Figure 15.13). The hydroxyl groups on the Tween molecules can be activated for coupling to biomolecules using many of the methods discussed for hydroxylic particles in Chapter 14. For instance, activation of Tween 20 with carbonyldiimidazole (CDI) or disuccinimidyl carbonate (DSC) in a nonaqueous solution provides a reactive derivative suitable for coupling to amine-containing molecules, such as proteins.

The following protocol for the activation of Tween 20 with CDI and its subsequent use in modifying a carbon nanotube and coupling an affinity ligand is based on the method of Chen *et al.* (2003).

Protocol

Activation of Tween 20

1. Dissolve 5 mg of Tween 20 in 25 ml of dry DMSO with stirring.
2. Add 4 g of CDI with mixing and react for 1 hour at room temperature.
3. Precipitate the CDI-activated Tween 20 by the addition of ethyl ether, and then isolate the precipitate using centrifugation or filtration. Redissolve the precipitated product in DMSO and repeat the precipitation process two more times to insure removal of excess reactants and reaction by-products. After the final precipitation, dry the isolated precipitate overnight *in vacuo* to remove remaining solvent.

Coating Nanotubes with CDI-Activated Tween 20

4. Suspend the SWNTs in 1 percent (w/w) CDI-activated Tween 20 solution in water using sonication and allow the detergent molecules to bind for 30 minutes at room temperature.
5. Quickly remove excess detergent by filtration on a 0.2 μm filter and washing the modified nanotubes with water.

Coupling of Activated Tween 20 to an Amine-Containing Ligand

6. Dissolve an amine-containing ligand in 0.1 M sodium carbonate, pH 9.5. If coupling a small ligand, such as a biotin-PEG-amine compound (Chapter 18), then use a concentration of about 5–10 mM in the carbonate buffer. For proteins, concentrations of 10 nM to 1 μM can be used with success.
7. Resuspend the activated nanotubes in the ligand-containing carbonate buffer and react with mixing overnight at room temperature or 4°C (e.g., for sensitive proteins).
8. Wash the coupled nanotubes with water or a suitable buffer using filtration and finally store them in buffer containing a preservative at 4°C.

In a similar approach to the noncovalent modification of carbon nanotubes with detergent molecules, Kam *et al.* (2005) used phospholipid derivatives to coat SWNTs for photothera-peutic agents against tumor cells *in vivo*. A phospholipid containing a PEG-NH_2 group was used to couple folic acid as an affinity ligand (using EDC to form an amide bond), which preferentially can be taken up by cancer cells. SWNTs were modified by the lipid derivative in aqueous solution using sonication for 1 hour and centrifuged to remove insoluble material. The aqueous fraction contained modified nanotubes, which contained the surface adsorbed lipid derivative. After the modified SWNTs were incubated with cells, the solution was irradiated

using an 808 nm laser. The nanotubes absorb light in this region of the spectrum and heat up to the point of causing cell death.

2.4. Pyrene Modification of Carbon Nanotubes

Another method for the noncovalent modification of carbon nanotubes involves the interaction of pyrene derivatives with the graphene sidewalls, presumably due to π-stacking (Nakashima *et al.*, 2002) (Figure 15.14). This interaction forms tight complexes that completely coat the nanotube surface, and if the pyrene contains a hydrophilic portion, it can impart water solubility to the SWNT, as well. The additional presence of reactive groups or functional groups on a pyrene side chain permits conjugation to affinity ligands for biological applications.

Nakashima *et al.* (2002) found that a pyrene derivative containing a single side chain terminating in a positively charged quaternary amine imparted water solubility to SWNTs treated with the compound. The application of other pyrene derivatives can be done to contribute both water solubility and an appropriate functionality for bioconjugation. Examples of pyrene derivatives that are suitable for the noncovalent modification of carbon nanotubes include those that have a single modification to the pyrene ring structure. Derivatives that contain multiple modifications off the pyrene group may affect its interaction potential for the SWNTs and should be avoided. For instance, water-soluble poly-sulfonated derivatives of pyrene, such

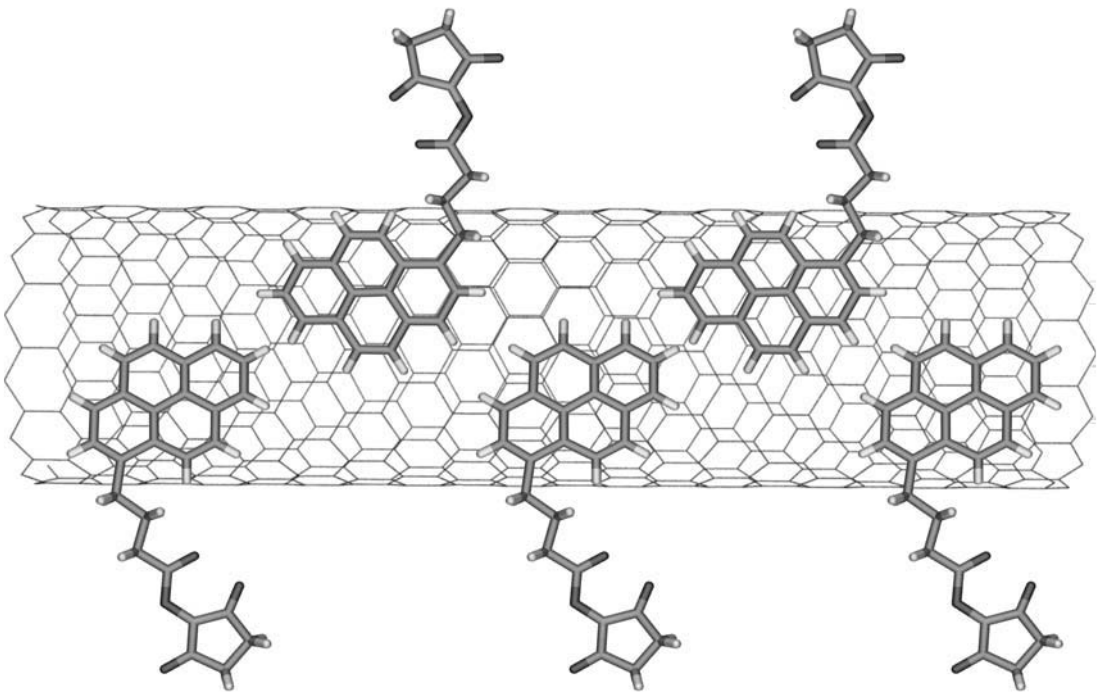


Figure 15.14 The NHS ester of a pyrene butyric acid derivative can be used to modify a carbon nanotube by adsorption of its rings onto the surface of the tube. The NHS ester groups then can be used to couple amine-containing molecules to form amide bonds.

as the Cascade Blue fluorescent dyes described in Chapter 9, Section 5, should not be used, because the pyrene structure is too hydrophilic to associate with the nanotube surface due to the three negative charges contributed by the sulfonic acids.

Some commercially available pyrene compounds that may be used to functionalize a carbon nanotube by this method include 1-pyrenebutyric acid and 1-aminopyrene (from Acros or Aldrich) as well as *N*-(1-pyrenyl)maleimide, 2-(1-pyrenyl)ethyl chloroformate, 1-pyrenebutyric acid *N*-hydroxysuccinimide ester, 1-pyrenecarboxaldehyde, and 1-pyreneacetic acid (from Aldrich). Each of these compounds provides a single site of derivatization off the basic pyrene rings to contain either a functional group or reactive group for coupling ligands. Molecules modified with these pyrene derivatives may be used to treat a carbon nanotube to form a stable noncovalent complex.

The pyrene derivatives containing a carboxylate group, chloroformate, aldehyde, or an NHS ester can be used to couple to amine-containing ligands, including proteins. The maleimide-pyrene derivative may be used to couple with thiol-containing ligands, while the amine–pyrene compound may be used to conjugate with carboxylate-containing ligands. Also available are (1-pyrenyl)butyric acid hydrazide and pyrene-1-isothiocyanate from Molecular BioSciences, which react with aldehydes and amines, respectively. Once a ligand is conjugated to a pyrene derivative, the complex may be incubated with a carbon nanotube to produce the final noncovalent complex. Since the initial modification is done on a water-insoluble nanotube, it is best to do the primary coating of the pyrene derivative in an organic solvent, such as DMF. It then is desirable to make the nanotube water-soluble by linking a hydrophilic spacer arm to the pyrene–nanotube complex. If a hydrophilic spacer is built into the resultant pyrene conjugate, such as the use of a short-chain PEG compound, then the resultant SWNT complex will be completely water-soluble (for example, see Figure 15.15). The PEG spacer chosen for this purpose should contain a terminal functional group for coupling to another molecule. At this point, the water-soluble complex can be reacted with a protein or other affinity ligand in aqueous buffer to make the desired bioconjugate. This multi-step process will result in a biocompatible carbon nanotube that retains its electronic properties, is water-soluble, and has added fluorescent properties due to the pyrene molecules coating its surface.

Maehashi *et al.* (2007) used pyrene adsorption to make carbon nanotubes labeled with DNA aptamers and incorporated them into a field effect transistor constructed to produce a label-free biosensor. The biosensor could measure the concentration of IgE in samples down to 250 pM, as the antibody molecules bound to the aptamers on the nanotubes. Felekis and Tagmatarchis (2005) used a positively charged pyrene compound to prepare water-soluble SWNTs and then electrostatically adsorb porphyrin rings to study electron transfer interactions. Pyrene derivatives also have been used successfully to add a chromophore to carbon nanotubes using covalent coupling to an oxidized SWNT (Álvaro *et al.*, 2004). In this case, the pyrene ring structure was not used to adsorb directly to the nanotube surface, but a side-chain functional group was used to link it covalently to modified SWNTs.

2.5. Modification of Carbon Nanotubes by Cycloaddition

The covalent methods previously discussed for fullerene modification using cycloaddition reactions also can be applied to carbon nanotubes. This strategy results in chemically linking molecules to the graphene rings on the outer surface of the cylinder, resulting in stable

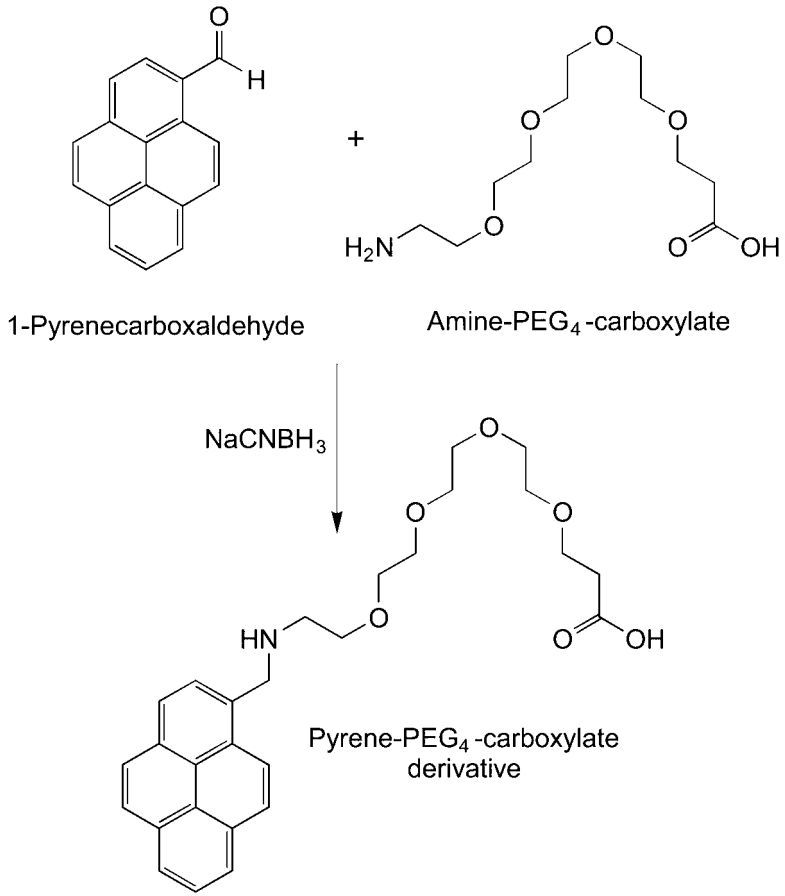


Figure 15.15 An aldehyde derivative of pyrene can be used to couple a hydrophilic amino-PEG-carboxylate spacer by reductive amination. The resultant derivative then can be used to coat a carbon nanotube through pyrene ring adsorption and result in a water-soluble derivative containing terminal carboxylates for coupling amine-containing ligands.

conjugates that can be designed to include hydrophilic groups for water solubilization. Georgakilas *et al.* (2002) describe the use of a 1,3-dipolar cycloaddition process to carbon nanotubes with azomethine ylides, generated by condensation of an amino acid derivative and an aldehyde. The reaction occurs in organic solvent at high temperature over a time period of several days.

Typically, SWNTs are suspended in DMF using sonication and the aldehyde and glycine derivatives are added to the mixture with stirring. The α-amine derivative of glycine can include hydrophilic spacers to make the resultant nanotube water-soluble as well as include protected functional groups to couple affinity ligands after deprotection (Kurz *et al.*, 1998). The aldehyde also can include R groups that add water solubility or functionality to the nanotube. A combination of a glycine derivative with an aldehyde derivative can result in both hydrophilicity and a functional group to conjugate ligands (Figure 15.16).

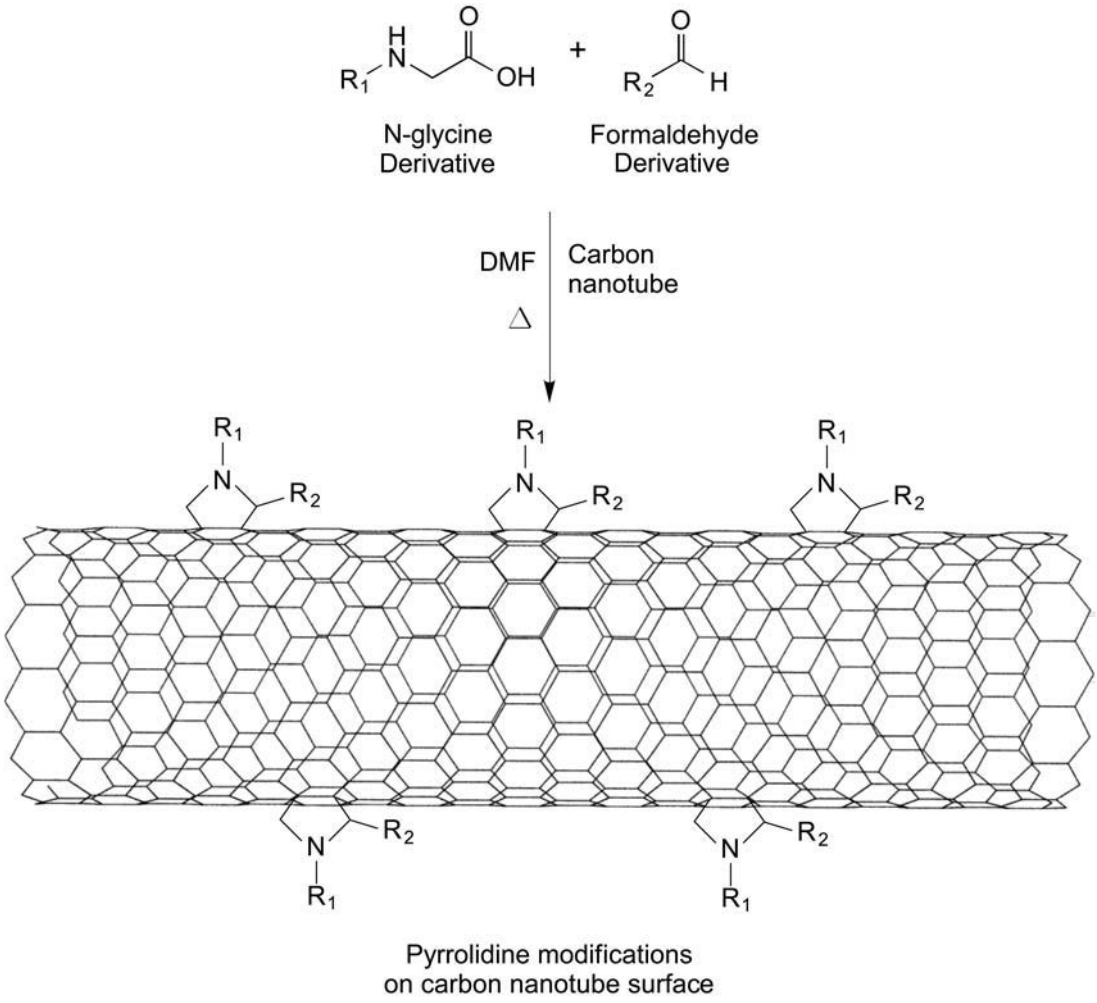


Figure 15.16 Some modification methods that are useful for fullerenes also can be used with carbon nanotubes. The reaction of an *N*-glycine compound with an aldehyde derivative can result in cycloaddition products, which create pyrrolidine modifications on the nanotube surface.

Felekis and Tagmatarchis (2005) used this cycloaddition process to prepare SWNT derivatives possessing photoactive components, such as the addition of ferrocene groups. They used a short PEG-type spacer on the glycine to impart water solubility at the same time.

Singh *et al.* (2006) also used cycloaddition to prepare carbon nanotubes containing indium labeled diethylenetriamine pentaacetic acid (DTPA) derivatives (Figure 15.17). In the initial modification, a SWNT was derivatized to contain a primary amine at the end of a short PEG spacer. The resultant water-soluble nanotube then was reacted with DTPA to create a metal chelating group at the end of the chain. Subsequent loading of the chelate with ^{111}In created a radionuclide–SWNT complex for *in vivo* biodistribution studies.

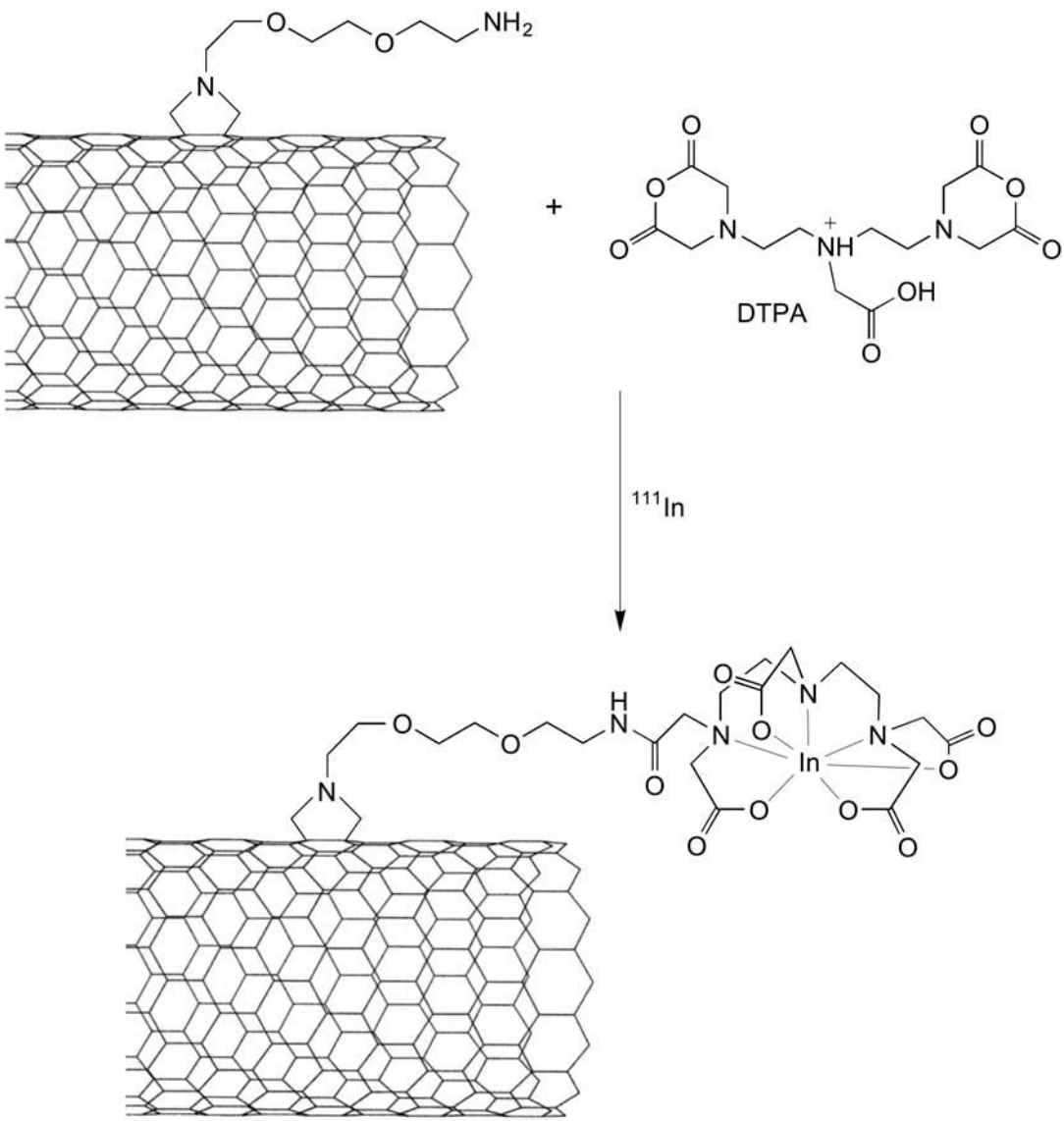


Figure 15.17 An amino-PEG-pyrrolidine derivative of carbon nanotubes can be used to couple metal chelating groups, such as DTPA. Subsequent coordination of ^{111}In results in an indium chelate that can be used for imaging applications.

The demonstration that the 1,3-dipolar cycloaddition process with azomethine ylides works with nanotubes implies that similar reactions developed for use with fullerenes also may be successful with carbon nanotubes. In particular, the cyclopropanation reactions discussed previously for the modification of C_{60}, likely will work for derivatization of SWNTs and MWNTs (Zakharian *et al*., 2005).

16

Mass Tags and Isotope Tags

Mass spectrometry has become one of the most important tools for analyzing proteins in complex biological samples. The ability to separate proteins and peptides in high resolution has made possible the simultaneous identification of hundreds of proteins within samples (for reviews, see Gingras *et al*., 2005; Hamdan and Righetti, 2005; Siuzdak, 2006). Proteins can be analyzed for their presence or compared between samples for their relative expression level. One cell population treated with a drug candidate, for instance, can be compared by mass spec to another sample as control to assess the affect of the drug on expression levels of certain proteins.

There are several ways that proteins can be analyzed using mass spec. Whole proteins can be separated using electrospray ionization (ESI) technique or by using matrix-assisted laser desorption/ionization (MALDI). Both of these methods inject intact proteins into the mass spectrometer, ionize them, and separate the resultant charged components by their individual mass/charge ratios. These methods work well for small and medium sized proteins in samples of low complexity, but analysis of larger proteins or highly complex samples is difficult. Alternatively, proteins first can be proteolytically digested using an enzyme such as trypsin and then the peptides analyzed by mass spec. This proteolysis method is more universally applicable, because analysis of peptide fragments allows mass spec separation to be done for all proteins regardless of their original intact mass prior to digestion. The peptides typically are subjected next to a higher energy secondary mass spec separation that fragments them into their component amino acids, which then can be identified by their masses. Samples then are analyzed by correlation of the peptide sequences to online databases of mass spec information, which can identify the protein that each peptide came from.

However, the interpretation of mass spec data on whole samples can be daunting, especially when analyzing proteolytically digested samples, which results in many times more species to analyze per protein than intact proteins. In order to reduce the complexity of sample analysis, a number of techniques have been developed to fractionate the proteome prior to mass spec separation. For instance, two-dimensional electrophoresis can separate proteins both by charge and by molecular weight and allow picking of only certain spots for subsequent mass spec analysis. However, two-dimensional electrophoresis is severely limited in its sensitivity for picking up low or medium copy proteins (Gygi *et al*., 2000). Alternatively, affinity separations on resins or surfaces can be done to capture only those proteins having certain epitopes or chemical characteristics, such as post-translational modifications. In addition, nanoliter HPLC

separations in one or two dimensions can be done to fractionate the peptides in complex samples by charge, size, or hydrophobicity before being injected into the mass spec.

Another major technique to simplify the analysis of protein samples is to use mass tags. Mass tags are modification reagents that contain a reactive group for coupling to biomolecules and another component of known mass, which behaves predictably upon MS separation. The mass tag also may contain a functional group for capture and separation on an affinity support, which permits further fractionation of the proteome. MS analysis of mass tagged peptides can be done by focusing only on those peptides that contain an additional mass component representing the tag's known mass contribution. Thus, all other peaks on the MS spectrum can be ignored, which greatly reduces the complexity of the sample. Mass tag reagents have been developed with reactive groups to modify specifically only certain low frequency amino acids within proteins. For instance, a thiol-reactive iodoacetyl group on a mass tag can be used to modify only those peptides having cysteine residues, thus removing from the analysis window all other peptides not containing cysteine.

The design of mass tags also can be combined with stable isotope labels to create more than one mass unit for each tag type (Schneider and Hall, 2005). For example, certain hydrogen atoms on one mass tag can be replaced with deuterium atoms on another derivative. Everything else on the tag is identical except for the isotope substitutions. Thus, the two mass tag analogs will differ in molecular weight by exactly the mass difference represented by the isotopic substitutions. Such tags can be used to modify a test sample with the stable isotope tag versus a control sample modified with the normal tag. If the two samples then are combined and analyzed by mass spec, their signal peaks generated from the tagged peptides will differ in mass units by the isotopic mass differences in the two tags. Identification of the peptides from both samples is done by looking for peptide peak pairs differing by the characteristic mass amount, therefore greatly reducing the complexity of sample analysis, and allowing simultaneous investigation of two samples. In this way, a test sample's protein expression levels can be compared to a control sample by measuring the different areas of the paired peptide peaks. The ability to analyze protein expression in two samples is vitally important to drug discovery and life science research applications studying the proteome.

Mass tags also can be broad spectrum in their modification properties to derivatize all peptides as they are formed upon proteolysis. For instance, one of the simplest mass tagging systems is to use the oxygen isotope ^{18}O in the water used during the enzymatic digestion of a protein sample (Miyagi and Rao, 2007). Upon hydrolysis by trypsin, the resultant C-terminal carboxylates that are formed each incorporate two ^{18}O atoms. Thus, peptides formed from ^{18}O digestion will be four mass units heavier than peptides formed by proteolysis using normal water. Mass spec analysis of this difference can identify the peptide pairs resulting from a control sample and a test sample run simultaneously.

Other broad-spectrum mass tag modification agents are designed to modify all amine groups and yield tags on every peptide at their N-terminal amines. For instance, small molecule tags using deuterium labeled forms and regular hydrogen labeled ones, such as the use of isotopically labeled propionic anhydride (Zappacosta and Annan, 2004), provide differentiation in the mass spec signals of peptides from test samples and controls. To eliminate interference, side chain lysine amines are blocked by guanidination with *O*-methylisourea hemisulfate and cysteine thiols are blocked with iodoacetamide (Leitner and Lindner, 2004). Some mass tag reagents of this type are able to differentiate peptides from 6 to 10 samples analyzed at the same time (see section on isobaric tags, this chapter).

The following sections describe some of the major mass tag types and discuss the general protocols for their use.

1. ICAT Reagents

Isotope coded affinity tags (ICAT) are bifunctional mass tagging agents containing a reactive group on one end of the molecule and an affinity capture group on the other end (Gygi *et al.*, 1999; Aebersold, 2003) (Figure 16.1). In addition, a portion of the tag can contain stable isotope substitutions, usually designed to be in the cross-bridge between the reactive group and the biotin handle. The original ICAT reagent contained eight deuterium atom substitutions on the outer ends of an ethylene oxide spacer. The isotope tagged version thus differs from its normal atom analog by exactly eight mass units.

Most ICAT style compounds contain a thiol-reactive iodoacetyl group on one end and a biotin handle on the other end of a spacer arm (Figure 16.2). Reagents of this type are highly specific for reacting with cysteine thiols in proteins to result in stable thioether modifications containing a terminal biotin group (Figure 16.3). After enzymatic digestion, modified peptides then can be isolated using immobilized (strept)avidin, which specifically binds only to those peptides containing the biotin tag and allows the other peptides to be discarded. Thus, the sample complexity can be reduced to analyze only peptides that contain a cysteine residue, which in the human proteome represents about 26.6 percent of the total tryptic peptides in a sample. This translates into the ability to cover 96.1 percent of all the proteins in the human proteome by targeting only cysteine-containing peptides (Zhang *et al.*, 2004; Yan and Chen, 2005).

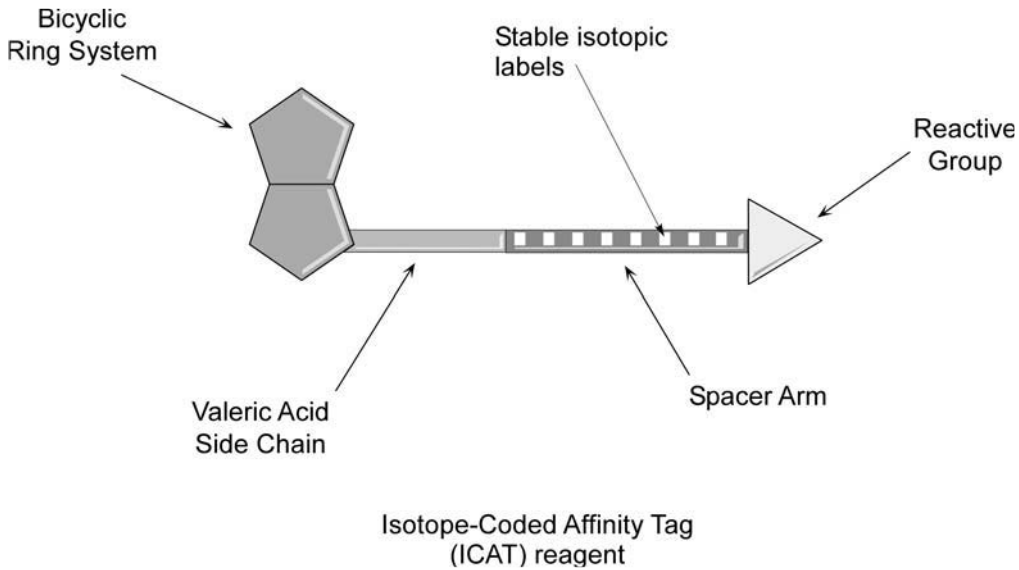


Figure 16.1 The general design of an ICAT reagent consists of a biotinylation compound with a spacer arm containing stable isotope substitutions. The reactive group is used to label proteins or peptides at particular functional groups and the biotin affinity tag is used to isolate labeled molecules using immobilized (strept)avidin.

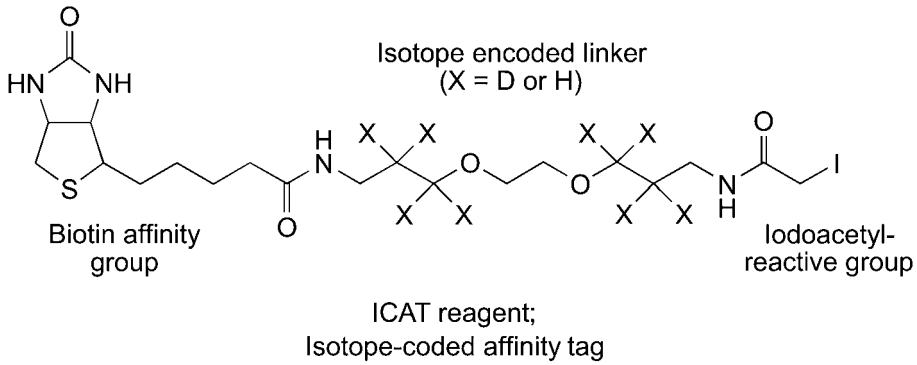


Figure 16.2 The original design of the ICAT compound. The iodoacetyl group provides reactivity with thiol groups. The isotopically labeled spacer arm typically is substituted with eight deuterium atoms.

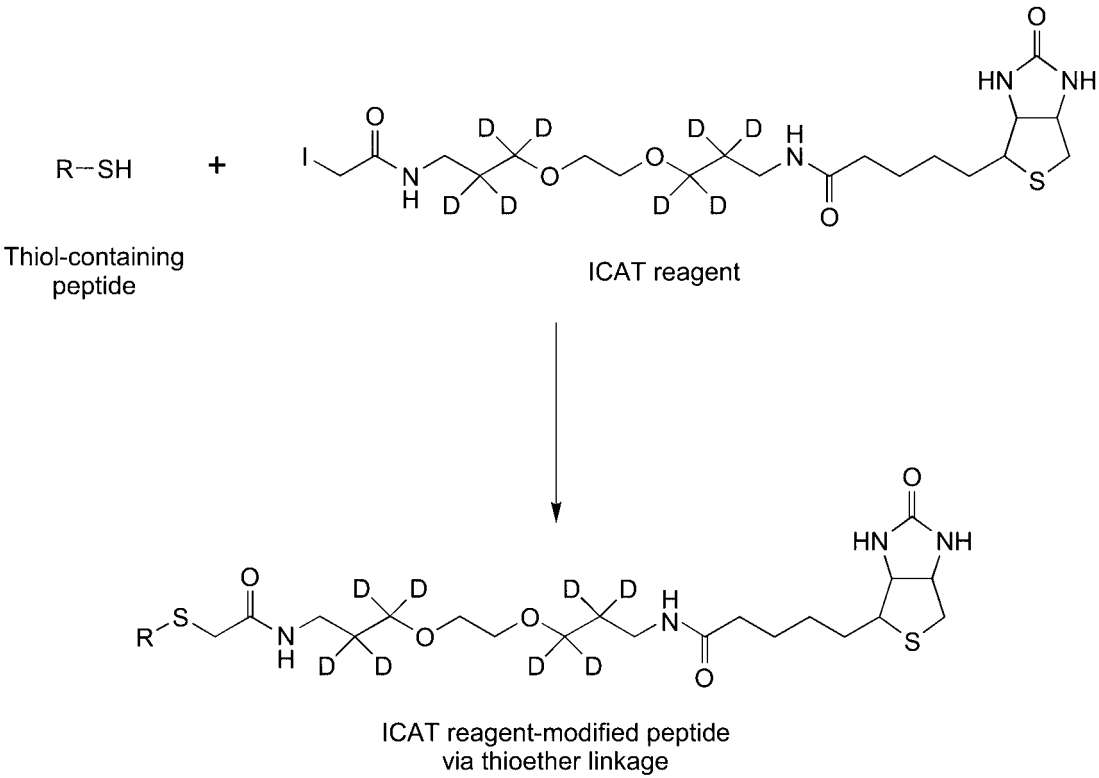


Figure 16.3 The ICAT reagent reacts with cysteine-containing peptides to form a thioether bond.

ICAT reagents can be used to compare two different samples by mass spec analysis. For instance, one cell population can be treated with a drug candidate, while another one remains untreated and acts as a control. Alternatively, one cell population can represent a disease state and the control population is the normal cell line. After cell lysis, the proteins in each

sample are denatured and reduced to make available all of the cysteine thiols for modification. One sample then is reacted with the heavy atom ICAT reagent, while the other sample is reacted with the normal isotope compound. The two samples next are combined and enzymatically digested with trypsin to generate peptide fragments, some of which will contain ICAT labeled cysteine groups. This combined peptide sample is affinity separated on an immobilized (strept)avidin column (or monomeric avidin column), which binds biotin labeled peptides from both sample populations equally. After removal of the non-biotinylated peptides by washing the column followed by elution of the ICAT labeled peptides, the sample is subjected to capillary reverse phase chromatography leading into ESI or MALDI mass spec analysis. The final HPLC separation again reduces the complexity of the sample set by further fractionating the peptides based on comparative hydrophobicity. In the MS spectrum, the relative peptide concentrations are determined by comparing all peaks separated by exactly the mass unit differential between the heavy atom mass tag and the normal atom mass tag. Each peptide sequence then is identified by fragmentation of the peptides into amino acid ions in a second dimension MS separation (MS/MS). Comparison of the amino acid sequence of each peptide peak to known sequence databases can identify the protein from which it came. Thus, the resultant peptide peak ratios are directly proportional to the relative amounts of the corresponding proteins present in the cell population.

The original ICAT design was found to have a number of deficiencies that often prevent the reagent from providing acceptable MS results. First, the deuterium isotope-labeled compound has a tendency to behave differently than the normal hydrogen isotope during reverse phase separation (Regnier *et al.*, 2002). If the labeled peptides that are identical except for the presence or absence of a D_8 ICAT modification don't elute at precisely the same point in an HPLC separation, then the MS analysis won't provide the peak pairs necessary for quantification. To solve this problem, a second-generation ICAT compound was designed containing ^{13}C isotopes instead of deuterium atoms. This type of reagent facilitates precise chromatographic separation of the labeled peptides and thus gives far superior performance upon MS analysis.

A second problem in the original ICAT design relates to the presence of the biotin tag. The biotinylated peptides often give undesirable fragmentation patterns during MS/MS analysis, which interferes with the smooth identification of peaks. Removing the biotin tag before mass spec analysis therefore would be beneficial to interpreting the MS results. Another issue with using a biotin tag is the elution step from the immobilized (strept)avidin column. Only under severely denaturing conditions is the interaction between biotin and (strept)avidin disrupted. However, even when using such conditions, the bound peptides do not always get released reproducibly from the column. The result is inefficient recovery of labeled peptides, which directly translates into a lack of precision in the MS data. Even using an immobilized monomeric avidin column does not completely solve this problem, because this affinity support sometimes has higher affinity binding sites or binds non-biotinylated peptides nonspecifically. To solve these issues, new cleavable ICAT designs were created that contain a bond within the cross-bridge that can be chemically broken (Li *et al.*, 2003). After binding to the (strept)avidin column, elution can be accomplished by cleaving the biotin arm, not by breaking the (strept)avidin–biotin interaction. The cleavage site can consist of a disulfide group within the cross-bridge (Turecek, 2002) that can be reduced for elution from the (strept)avidin column or it can consist of an acid cleavable linker arm (e.g., a carbamate bond) within the ICAT structure (Fauq *et al.*, 2006) (Figure 16.4). Either method dramatically improves the recovery of labeled peptides from the affinity column and thus provides increased precision in the samples leading

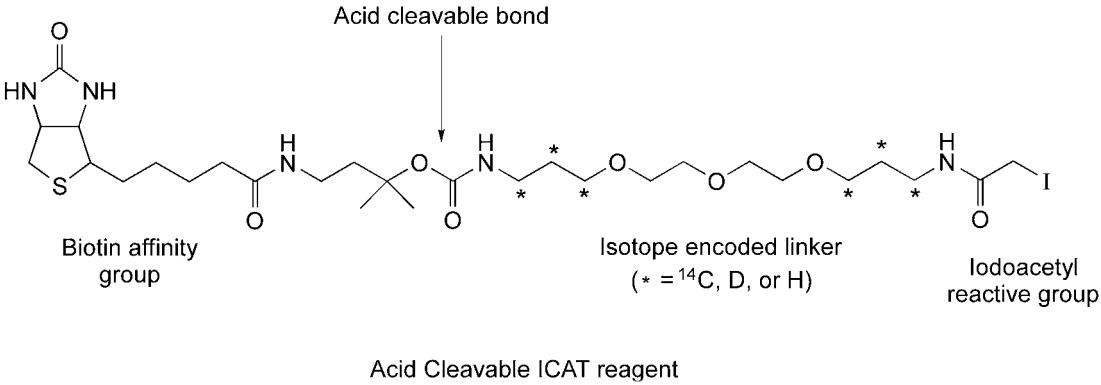


Figure 16.4 A more advanced ICAT design uses an acid-cleavable spacer arm to facilitate elution of labeled peptides from a (strept)avidin affinity column. The use of ^{14}C isotopes instead of deuterium labels permits precise reverse phase separations prior to mass spec that show no elution peak time differences between isotope-labeled and normal atom-labeled peptides.

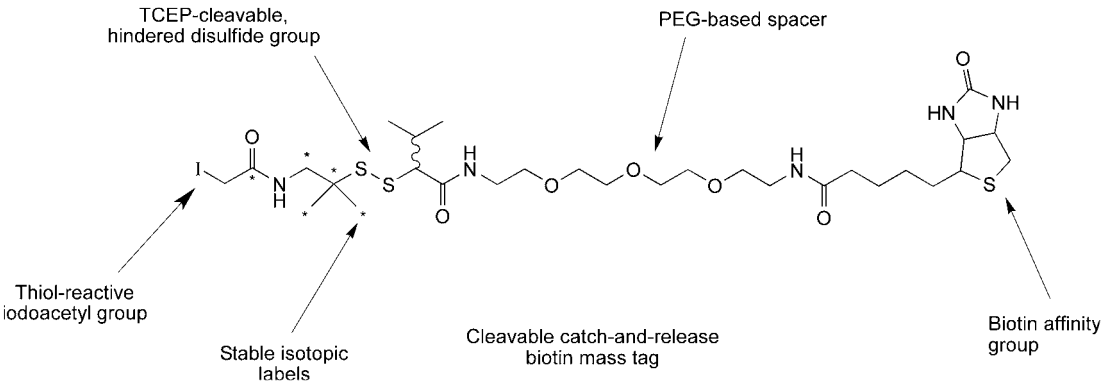


Figure 16.5 A catch-and-release ICAT design incorporates a gem-methyl group and an isopropyl group on either side of a disulfide bond within its spacer arm. The hindered disulfide permits the use of standard reducing gel electrophoresis conditions using DTT without reduction. After purification on a (strept)avidin affinity column, however, the disulfide group can be cleaved with TCEP, which provides recovery of the labeled peptides prior to mass spec separation.

into the LC–MS analysis. The ^{13}C labeled, acid cleavable ICAT reagent has been used to identify successfully low-level protein expression in highly complex samples (Hansen *et al.*, 2003).

Another new ICAT design, termed a "catch-and-release" tag, contains a constrained, sterically hindered disulfide linkage with bulky alkyl groups on both sides. The hindered nature of the disulfide makes it stable to standard protein reduction procedures, but it can be specifically reduced upon the addition of tris(2-carboxyethyl)phosphine(TCEP) (Gartner *et al.*, 2007) (Figure 16.5). This allows proteins to be labeled with the catch-and-release ICAT compound that have undergone reduction using dithiothreitol (DTT) to cleave protein disulfides but not affect the disulfide group in the reagent cross-bridge. Only after capture of labeled peptides on

a (strept)avidin column is the cross-bridge cleaved by the addition of TCEP and the labeled peptide recovered.

A variation on the ICAT mass tag concept was made by immobilizing the label on a solid phase (Zhou *et al*., 2002). Using this design, cysteine-containing peptides are modified directly on a beaded insoluble support. After washing away non-cysteine peptides, the linked peptides can be cleaved from the matrix by use of a photo-cleavable group and eluting off the peptides with an isotope tag modification. This approach results in cleaner and more efficient isolation of tagged peptides and simplifies the ICAT labeling process (Figure 16.6).

Another novel mass tag design involves a spectrally visible ICAT variant developed to include a fluorescent group for detection purposes (Lu *et al*., 2004). Like the original ICAT reagent, the VICAT compound includes a thiol-reactive iodoacetyl group, a cleavable cross-bridge, an isotopically labeled portion, and a biotin handle. It also has another arm, however, that contains the chromogenic label, which is detectable by absorption at 493 nm and emission at 503 nm.This group allows detection of peptides in samples separated by chromatographic or electrophoretic means. Quantification of the fluorescent tag in isolated peptides can provide absolute information regarding the level of proteins present in a cell.

ICAT type compounds are designed to enrich for peptides containing one particular amino acid residue, usually cysteine. The affinity capture step removes other non-cysteine peptides and thus reduces the complexity of the MS data set. ICAT reagents also can be designed with a different reactive group that is able to covalently couple to other amino acid groups (or even sites of post-translation modification) to change the selectivity of the peptide population being analyzed. However, it is best to target amino acids or functional groups present in limited amounts within proteins, otherwise the tag may capture more peptides than could be conveniently measured by mass spec. Han *et al*. (2007) developed a hydrazide-ICAT compound to identify proteins modified by 2-alkenals derived from lipid peroxidation (LPO). This type of mass tag should be useful for the study of other oxidative changes on proteins, such as those resulting in aldehyde or ketone modifications (see Chapter 1, Section 1).

The following protocol describes the use of an acid-cleavable ICAT reagent, currently available from Applied Biosystems. This is not meant to be a detailed method describing every aspect concerning the use of mass spectrometry, but only to describe the modification reaction of the ICAT compound with proteins.

Protocol

1. Grow cells to 70–80 percent confluence and harvest by scraping the cells into 5 ml PBS, 5 mM EDTA, pH 7.4. Aliquot cell counts of approximately 2.5–5 $\times$ 10^6 for processing. Lyse cells using a detergent lysis buffer (e.g., Poppers, Thermo Fisher) or by mechanical means. Centrifuge and discard the cellular debris. Measure the total protein concentration using the BCA assay (Thermo Fisher) and adjust the protein concentration to 1.5 mg/ml using 50 mM Tris, 0.1 percent SDS, pH 8.0. Separately process a test sample and a control sample made up of different cell populations.
2. Reduce disulfides in the two protein samples by the addition of 2 µl of 50 mM TCEP (Thermo Fisher) to each 100 µl aliquot of protein solution. Cover and boil the samples for 10 minutes in a water bath to completely denature and reduce the proteins. Avoid the use of thiol-containing reductants, such as DTT, as these will react with the iodoacetyl group on the ICAT compound.

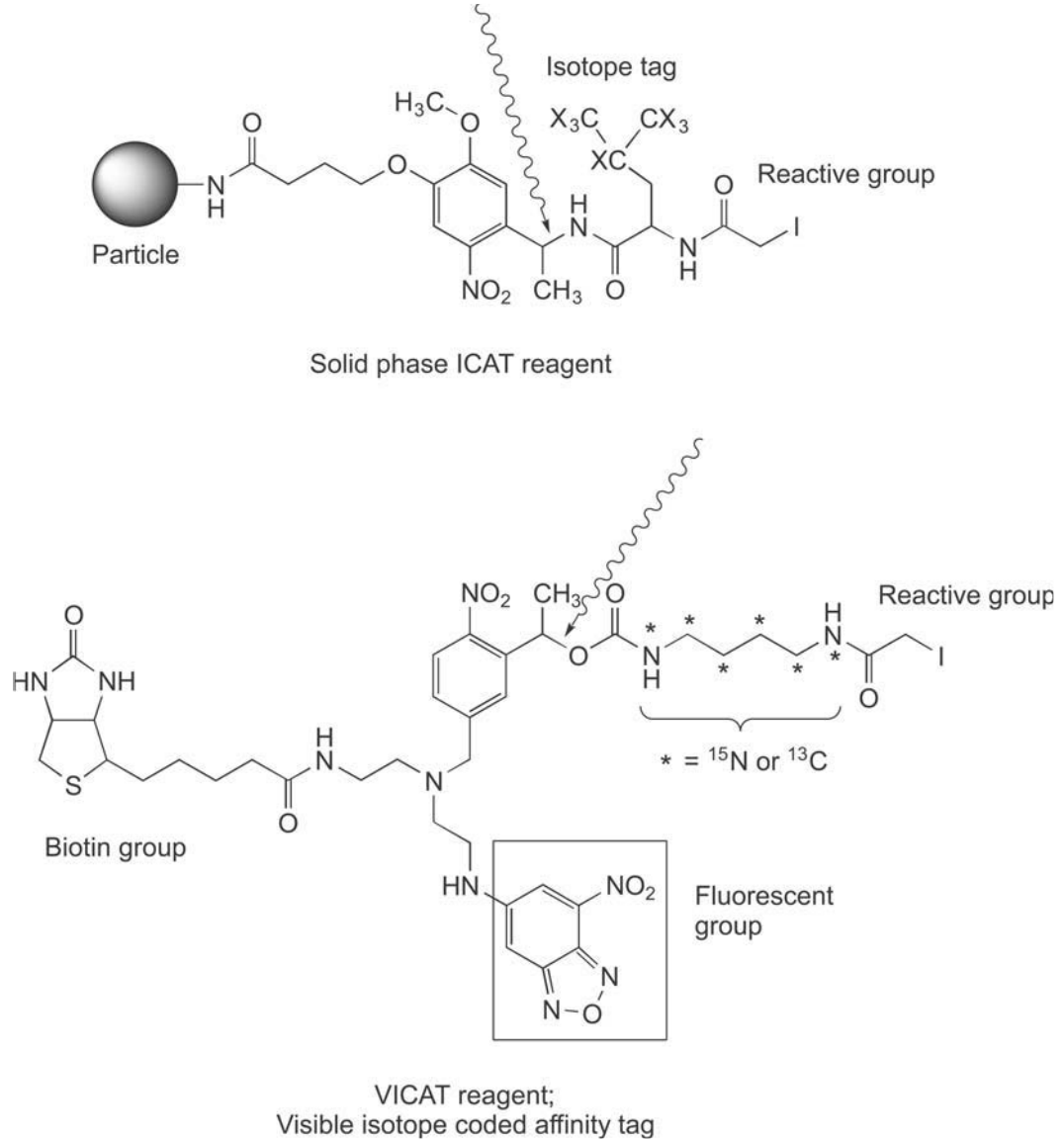


Figure 16.6 The solid phase ICAT reagent provides a thiol-reactive iodoacetyl group to capture cysteine peptides, a spacer containing stable isotopic labels, and a photo-cleavable group that can release the captured peptides for mass spec analysis. The VICAT mass tag is a solution phase labeling agent that also has a photo-cleavable site to release isolated peptides from a (strept)avidin affinity resin. This compound adds a fluorescent group to better detect labeled peptides as they are being isolated from a sample.

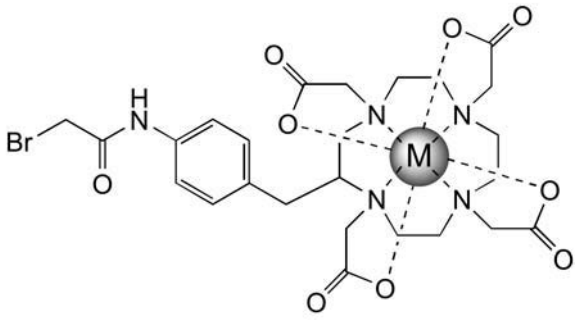
3. Dissolve one vial of heavy isotope, cleavable ICAT reagent (Applied Biosystems) in 20 µl acetonitrile (use a fume hood for handling solvents). Dissolve a second vial containing the normal isotope ICAT compound in 20 µl acetonitrile. Vortex mix each vial to dissolve.

4. Add 100 μg of the control protein solution to one vial of dissolved normal isotope ICAT reagent. Mix to dissolve. Add 100 μg of the test protein solution to one vial of dissolved heavy isotope ICAT reagent. Mix to dissolve.
5. React both solutions for 2 hours at 37°C.
6. Combine the test sample with the control sample in a single vial. Mix well.
7. Prepare a solution of TCPK-trypsin in 50 mM ammonium bicarbonate, pH 8.0, at a concentration of 100 ng/μl. Add 10 μl of the trypsin solution to every 10 μg of combined, labeled protein solution from Step 6. Incubate at 37°C for 12–16 hours or overnight with mixing.
8. Centrifuge the digested peptide mixture to remove any insoluble material. The biotinylated peptides then are purified on 20 μl immobilized monomeric avidin column. The column first is primed with elution buffer (0.4 percent TFA in 30 percent acetonitrile) and then washed with binding buffer (100 mM ammonium bicarbonate, pH 8.0). The sample is applied and washed through with binding buffer until all not-bound peptides are completely removed. The acetonitrile/TFA elution is subsequently used to cleave off the biotin group from the eluted peptides. *Note*: Additional fractionation may be done to reduce further the complexity of the sample, such as the use of ion-exchange chromatography. The eluted, labeled peptides finally are analyzed by LC–MS/MS.

2. ECAT Reagents

Element-coded affinity tags represent a new type of isotope tag for mass spec analysis (Corneillie *et al*., 2003, 2004; Whetstone *et al*., 2003; Meares *et al*., 2007). This system uses a bifunctional metal chelate group that securely coordinates a lanthanide metal ion. A reactive group also is present for the modification of certain amino acids in proteins, which typically consists of a bromoacetyl group. This group reacts similarly to an iodoacetyl group and forms thioether linkages with cysteine thiols. The ECAT design includes a DOTA chelating group (Chapter 10) containing four nitrogen atoms and four carboxylates to complex with any of the lanthanide series metal ions via eight coordination bonds. Simply by using different lanthanide elements within the complex the result will be a unique set of isotope tags having different mass signatures by MS. At least in theory, up to 15 different ECAT mass tag compounds could be created by using all of the different lanthanide metals representing elements 57–71. In addition, the lanthanides are naturally mono-isotopic in that they occur mainly in nature with only a single isotope. Only cerium contains a high percentage of another isotope in nature (88 percent ^{140}Ce and 11 percent ^{142}Ce), all the other lanthanides are >97 percent a single isotope. This is important for mass spec separations, as the resultant peaks won't contain extraneous mass signatures due to multiple isotopes (Figure 16.7).

The ECAT reagent bromoacetamidobenzyl-DOTA (BAD) can be used like an ICAT tag to label only those peptides containing cysteine residues (Figure 16.8), thus reducing the total number of peptides having to be analyzed in a mass spec separation. Unlike the ICAT reagent design, the ECAT compound does not contain a biotin handle for affinity separation. Instead, a monoclonal antibody has been developed with specificity for the DOTA chelate containing a bound lanthanide metal. The antibody will recognize any lanthanide element bound in the chelate and thus function as an affinity ligand for separating ECAT-labeled peptides. The affinity of the monoclonal for ECAT chelate structure allows highly stringent washes to be done



(S)-2-(4-(2-bromoacetamido)benzyl)-DOTA
Lanthanide metal chelate
Mol. Wt.: 738.67
(without Lanthanide)

Figure 16.7 The ECAT mass tag consists of a DOTA metal chelate group that can coordinate a lanthanide metal ion and a bromoacetyl group for coupling to cysteine-containing proteins.

prior to elution of the labeled peptides. The affinity column typically is washed with low pH, high salt, high pH, and 30 percent acetonitrile before elution is done. This removes all traces of nonspecifically bound peptides, so they can't interfere with the mass spec analysis. The ECAT labeled peptides then are eluted with 20 percent acetonitrile containing 0.1 percent TFA.

The ECAT system has an advantage over ICAT reagents in being available with more mass signatures than is achievable using ^{13}C or ^{2}H labeled tags, which are difficult to synthesize. In addition, it is not hampered by the binding idiosyncrasies of a biotin group interacting with (strept)avidin or the fragmentation problems a biotin tag gives on MS analysis. The ECAT chelate does not generate fragmentation products during the mass spec analysis. Also, the mass defect characteristic of lanthanide metals results in a mass signature upon MS separation that occurs in a relatively unoccupied region of the m/z spectrum (Schneider and Hall, 2005). Thus, identification of ECAT labeled peptides potentially is simpler than using ICAT reagents.

Another variant of ECAT reagent technology has been developed to analyze the products of protein oxidation (Lee *et al.*, 2006). Called "oxidation-dependent, carbonyl-specific element coded affinity tag" (O-ECAT), the compound contains the same DOTA lanthanide chelating group, but instead of a thiol-reactive bromoacetyl group, it has an aldehyde- or ketone-reactive aminoxy group (Figure 16.9). The aminoxy functional group can covalently link to aldehydes or ketones to give an oxime bond, which is stable under aqueous conditions (Figure 16.10).

The O-ECAT reagent is a superior alternative to the use of 2,4-dinitrophenylhydrazine (DNPH; Chapter 1, Section 1.1) in the study of protein oxidation. DNPH modification produces detectable complexes, but it does not provide information as to what amino acids are involved. O-ECAT modifies carbonyl end products of protein oxidation and in addition, it can provide exact information as to the amino acids that were oxidized. Mass spec analysis of modified proteins performed after proteolysis gives the exact amino acid sequences including the sites of O-ECAT reagent modification. The same antibody that is specific for the metal chelate portion of the standard ECAT reagent also can be used to capture and detect the O-ECAT

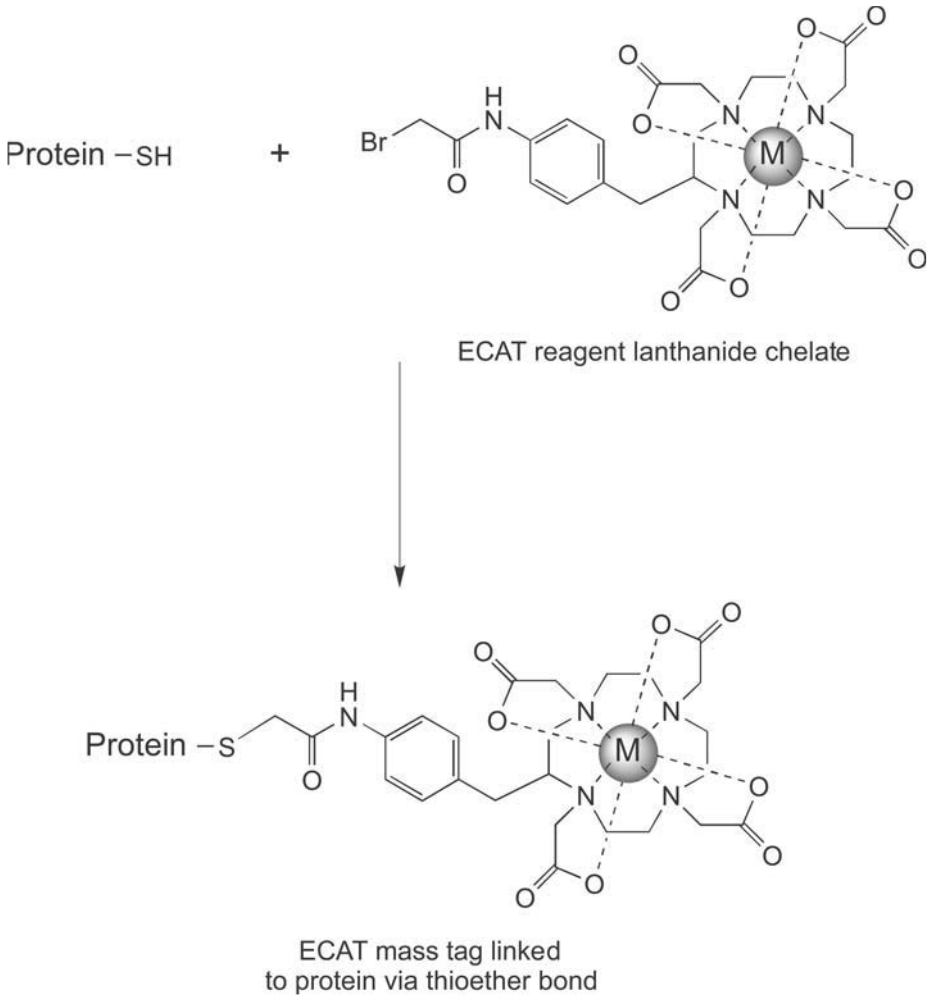
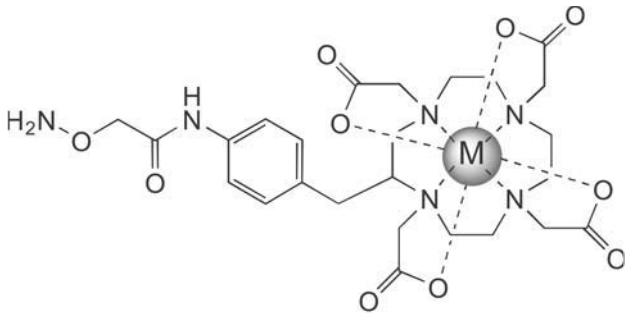


Figure 16.8 Reaction of the ECAT reagent with a cysteine-containing protein results in the formation of a stable thioether bond.

labeled proteins or peptides. Thus, O-ECAT-modified proteins can be detected in Western blots or the sites of oxidation quantified using ELISA-based assays.

3. Isobaric Tags

The use of mass tagging reagents to analyze proteomic data has greatly improved the ability to compare samples for protein expression differences. However, a major limitation of the ICAT procedure (Section 1, this chapter) is that it can only compare two samples simultaneously, usually a test and a control. Even with the ECAT design (Section 2) using multiple lanthanide metals to make a series of different mass tag signatures, it is difficult to extend the



O-ECAT Reagent;
(((S)-2-(4-(2-aminooxy)-acetamido)-
benzyl)-1,4,7,10-tetraazacyclododecane-
N, N', N'', N'''-tetraacetic acid

Figure 16.9 The O-ECAT reagent structure contains a DOTA chelating group and a terminal aminoxy group for coupling to aldehyde and ketone sites of oxidation within biological molecules.

analysis to multiple samples, because of the shear number of peaks that result in the mass spec separation.

A new type of mass tag extends the benefits of stable isotopic labeling of peptides for mass spec to the analysis of multiple samples and multiple proteins separated simultaneously within the same mass spec run. An isobaric tag consists of a reactive group for coupling to peptides followed by an isotopically labeled mass normalization group, a cleavable linker, and another isotopically labeled group, called a mass reporter (Figure 16.11). For every isotopic substitution in the mass reporter region, the mass normalization group has an inverse mass substitution. Using this balancing process, the total molecular mass of the entire tag always stays the same–thus the name "isobaric tag". A series of isobaric labels can be created by careful selection of the isotopic substitutions in the reporter group, which are exactly balanced in the normalization group.

If there are enough potential isotopic substitution sites in the isobaric tag design, a set of 4–10 tags can be created in which each has a different reporter mass, but all of them have the same total molecular weight. Proteome Sciences, Applied Biosystems, and PerkinElmer/Agilix each have commercialized isobaric tag sets based on the reporter group/normalization group blueprint for multiplexed sample analysis. Figure 16.12 shows examples of isobaric tag design, which were obtained from company advertising, scientific publications, or the associated patents. Most of the tags also contain a tertiary amine group or a guanidino group to aid in the ionization of the labeled peptide and provide better mass spec signals.

In use, a protein sample first is proteolytically digested and labeled with an individual isobaric tag. The most common isobaric tag design contains an amine-reactive NHS ester group to label each peptide at its N-terminus. Therefore, within a given sample all of the different peptides after labeling will have a unique isobaric tag modifying them with a characteristic reporter group mass. Unlike the use of an ICAT tag that targets only cysteine-containing peptides, modification of the N-terminus of every peptide assures 100 percent coverage of the proteome. If multiple samples are being analyzed, then each peptide sample is separately labeled

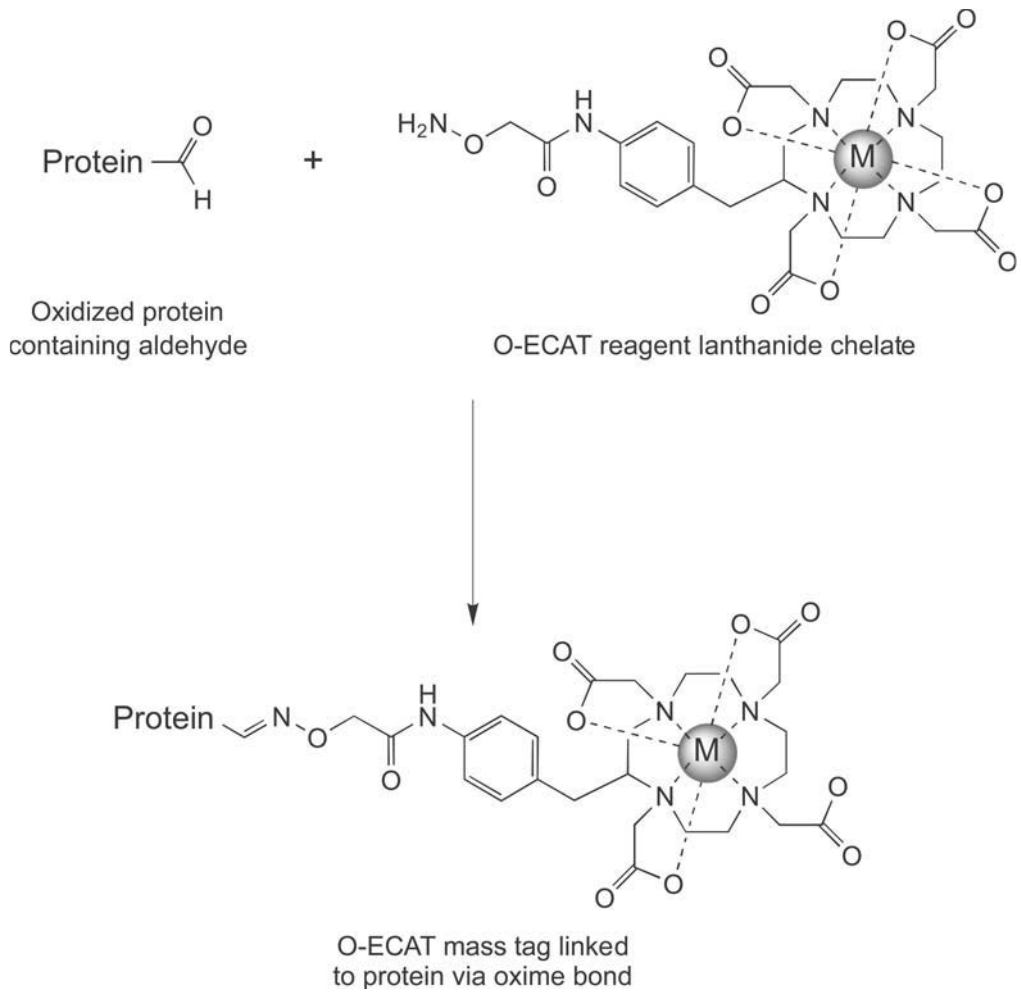


Figure 16.10 The O-ECAT mass tag can covalently link to any oxidized proteins containing aldehydes, forming an oxime bond.

with a different isobaric tag in the series. After labeling, all the samples then are combined and separated chromatographically to reduce the total sample complexity.

Since each isobaric tag in a set is structurally identical except for its isotopic substitution pattern, all of them perform identically with regard to peptide modification and chromatographic separation. Multiple peptide samples then can be labeled separately with different tags in a series, the samples combined, subjected to fractionation typically done by multidimensional protein identification technology (MudPIT; Liu *et al.*, 2004), and injected into a mass spectrometer for analysis. As each peak comes off the MudPIT separation system and goes into the mass spec, it contains the same sequences of labeled peptides from each sample that happen to elute under the instant conditions of salt strength and solvent addition being used at that moment. The only difference in the peptide mixture within a given chromatographic peak,

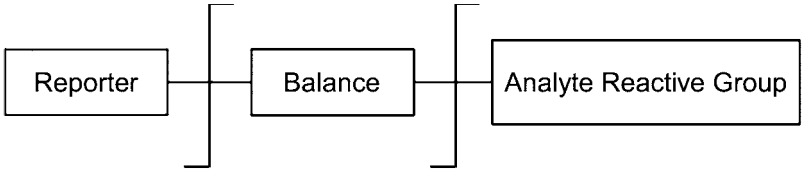


Figure 16.11 The general structure of an isobaric mass tag reagent. The reactive group facilitates coupling to discrete sites on peptides, such as amines. The reporter group creates a unique mass signal in MS^2 analysis and its total mass is exactly balanced by the balance group by changing the stable isotopic labels to provide the opposite mass differential as that of the reporter group. The result is that all isobaric tags have the same initial molecular mass, but upon fragmentation in MS^2, the reporter group is released and it provides the unique mass signal to identify the sample being analyzed.

then, is the type of isobaric label attached to them, which is indicative of the sample it came from. Thus, the peptides representing a particular sample will all be labeled with a tag having a characteristic reporter group mass. Another set of peptides with the same amino acid sequence but coming from another sample will be labeled with a different isobaric tag having another reporter group mass signature.

It is only upon tandem mass spec analysis that the isobaric labels can be distinguished and the peptides identified. For instance, if four samples were each labeled with a different isobaric tag, in the first dimension of a MS separation a given peptide from each sample will appear in the same peak, because the peptides will all have the same sequences and the isobaric tags labeling them will all have the same mass signatures. Therefore, their mass/charge ratios will all be the same and they will be indistinguishable at this point. However, upon MS/MS separation wherein additional energy is used to promote fragmentation of the labeled peptides within the peak (usually by collision-induced dissociation (CID) or electron capture dissociation (ECD)), the peptides will break down into their respective charged amino acids and the isobaric tags will be cleaved to release their reporter groups. From the peaks that result from this second stage MS separation both the peptide sequence and the sample from which it came can be identified from the amino acids and the mass of the respective reporter groups.

There are many publications describing the development and use of isobaric tags. For instance, Thompson *et al.* (2003) describes the development of tandem mass tags based on an isotopically labeled peptide design. A later iteration of this concept uses commercially available isotopes of alanine to form the reporter group, followed by a piperazine ring (as the charge-carrying group), a second alanine used as the mass normalization group, and a proline residue, which functions as the electrospray cleavable linker (Thompson *et al.*, 2007). Ross *et al.* (2004) used a set of four isobaric tags designed around an *N*-methyl piperazine group to study the global protein expression of a wild-type yeast strain versus strains defective in certain pathways. These tags are part of the iTRAQ (isotope tags for relative and absolute quantitation) system from Applied Biosystems. Figure 16.12 shows some of the major structural characteristics of commercial isobaric tags. Figure 16.13 illustrates the design of a multiplex isobaric set that uses a piperidine ring as the reporter group, showing all of the various isotopic modifications done to balance the reporter and normalization group to create six unique tags (TMT system: Tandem Mass Tags from Proteome Sciences).

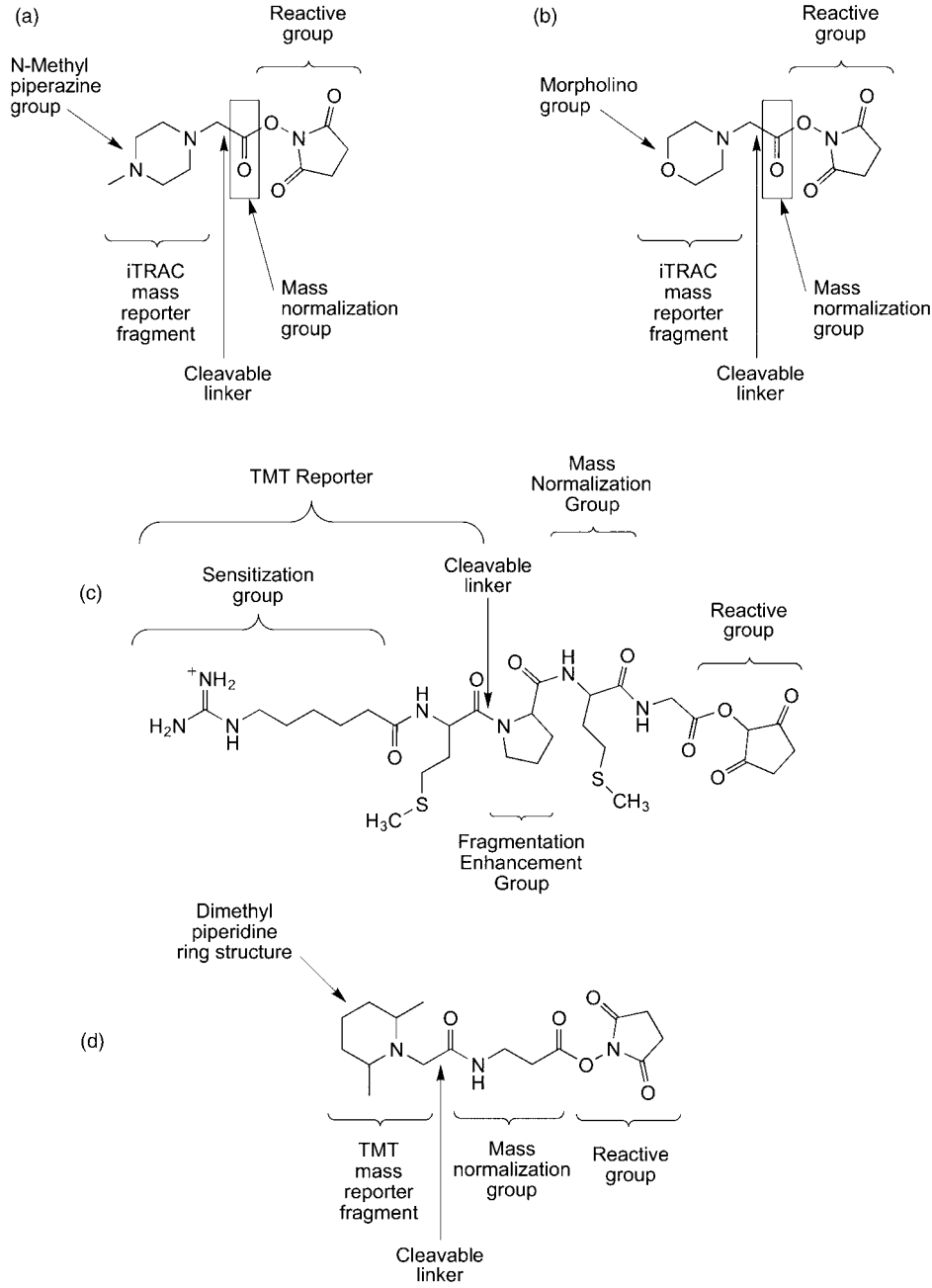


Figure 16.12 A number of isobaric tags have been developed having different structural motifs. (a and b): Isobaric tags used as part of the iTRAC reagents (Applied Biosystems). (c) An early design of isobaric tags using a core of amino acid derivatives (Proteome Sciences). (d) The TMT tag design as commercialized by Proteome Sciences. All of these isobaric tags contain a sensitization group to enhance mass spec detection, which is represented either by a tertiary amine that can be protonated to carry a positive charge or a guanidino group.

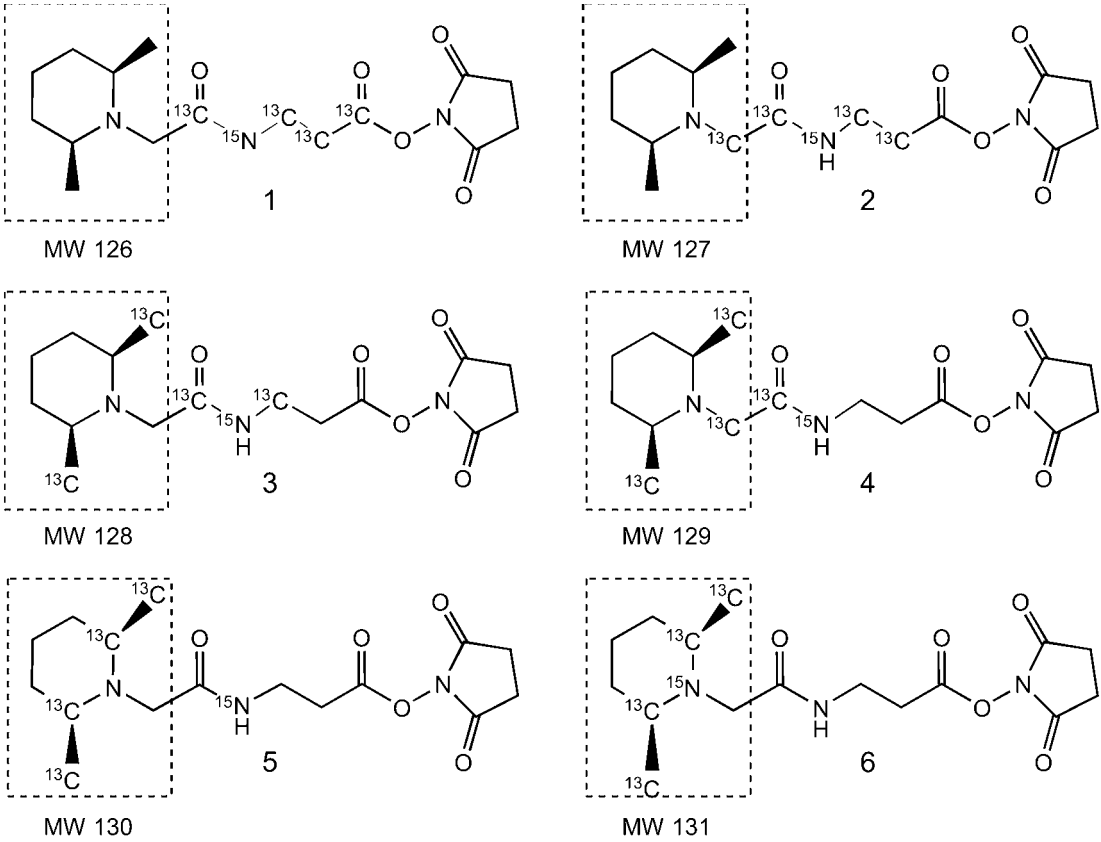


Figure 16.13 Isobaric tags allow multiplexed analysis of different samples by changing the mass of the reporter group and balancing that change by the opposite change in the balance group. This figure illustrates the 6-plex tag system from Proteome Sciences, which contains six different reporter groups (boxed areas). All of the reagents contain amine-reactive NHS esters for modifying lysine side chains in proteins and peptides.

Isobaric labels thus permit quantitative information regarding protein expression levels in multiple samples analyzed simultaneously by MS. The multiplexed capability of these reagents allows the measurement of peptides and proteins in diseased samples, treated samples, and normal samples all in the same experiment. In addition, since all peptides from a given protein get labeled at their N-termini, the MS analysis generates more than one peptide signal, which can be used to confirm protein identity with greater confidence than using a cysteine label, like ICAT.

The protocol for using isobaric tags differs from that described previously for the ICAT or ECAT type reagents. In the following method, the proteins are denatured and the disulfides reduced and then alkylated to block them permanently. This eliminates disulfide re-association and also prevents the isobaric tags from forming thioester modification with cysteine thiols. Next, the proteins are digested with trypsin and then modified with an isobaric tag. Each sample is labeled with a different isobaric compound so that the samples can be differentiated upon MS/MS analysis.

The following protocol illustrates the modification reaction and the handling of the protein samples, but it is not meant to be instructive of mass spec techniques.

Protocol

1. Grow cells to 70–80 percent confluence and harvest by scraping the cells into 5 ml of 0.1 M sodium borate, pH 7.5. Avoid the use of amine-containing buffers, as these will react with the NHS esters on the isobaric tags. Aliquot cell counts of approximately 2.5–5 $\times$ 10^6 for processing. Lyse cells using a detergent lysis buffer (e.g., Poppers, Thermo Fisher) or by mechanical means. Centrifuge and discard the cellular debris. Measure the total protein concentration using the BCA assay (Thermo Fisher) and adjust the protein concentration to 1 mg/ml using 0.1 M sodium borate, 0.1 percent SDS, pH 7.5. A total of 100 μg of each protein sample can be used in this protocol. Separately process test samples and a control sample made up of different cell populations. Simultaneous measurement can be done on a total number of samples equal to the number of different isobaric tags available.
2. Reduce disulfides in the protein sample by the addition of 2 μl of 50 mM TCEP (Thermo Fisher) to each 100 μl aliquot of protein solution (final concentration 1 mM). Cover and boil the samples for 10 minutes in a water bath to completely denature and reduce the proteins. Alternatively, reduction may be done at 60°C for 1 hour. Avoid the use of thiol-containing reductants, such as DTT, as these will react with the thiol blocking agent used in the next step.
3. Add 6 μl of iodoacetamide to each sample solution and react with mixing for 30 minutes at room temperature.
4. Prepare a solution of TCPK-trypsin in 0.1 M sodium borate, pH 7.5, at a concentration of 400 ng/μl. Add 10 μl of the trypsin solution to each sample and incubate at 37°C for 12–16 hours or overnight with mixing.
5. Dissolve the isobaric tagging reagents in acetonitrile or ethanol at a concentration of 50 mM (or according to the manufacturer's recommendations). Use a fume hood to handle organic solvents.
6. Add a quantity of the appropriate isobaric tag solution to each sample to provide a final concentration of 10–20 mM. This quantity of reagent will assure a large molar excess of reagent over the concentration of peptides present in order to modify completely all peptides at their N-terminus. Note that the ϵ-amino groups of lysine residues also will be modified by this procedure. React for 1 hour at room temperature.
7. To eliminate acylation products at tyrosine residues, add 1 μl of 15 M hydroxylamine solution in water to each protein sample and incubate for 30 minutes at 37°C (Zappacosta *et al*., 2006).
8. Combine the contents of each labeled sample into a single tube and mix by vortexing, then centrifuge.
9. Before LC–MS/MS analysis, the sample must be cleaned up to remove excess salts, SDS, reducing agent, and cell lysis buffer components. This typically is done by using a strong cation exchange matrix. Protocols may be found for this procedure in the instruction booklets related to the use of isobaric tagging reagents (e.g., Proteome Sciences, Applied Biosystems, or Perkin Elmer/Agilix).

17

Chemoselective Ligation: Bioorthogonal Reagents

The many dozens of reactions that are available for bioconjugation purposes generally are designed to work with biological molecules and the functionalities they contain. The main goal of most bioconjugate techniques is to use the functional groups on biomolecules to label with another type of biomolecule or link to synthetic probes. However, it is often desirable to couple one molecule to another without the potential for cross-reactivity with biomolecules. The term "chemoselective ligation" has been coined to describe the coupling of one reactive group specifically with another reactive group without side reactions in aqueous solution or in the presence of biological material (Lemieux and Bertozzi, 1998).

Unfortunately, the incredible diversity of biomolecules in cells and organisms presents problems for this goal of total chemoselectivity and bioorthogonality, as the number and variety of reactive sites on the molecules of life is extraordinary. Even the best crosslinkers or labeling reagents designed to be somewhat site-specific in their reactions often display cross-reactivity with functional groups on biomolecules other than the ones intended for coupling. For instance, *N*-hydroxysuccinimide (NHS) esters usually are considered amine reactive, but they also react with cysteine, histidine, serine, threonine, and tyrosine side-chain groups. Maleimide groups too are touted as being thiol specific, but amines also can react with maleimides given the right conditions.

Bioorthogonal reagents ideally should contain a reactive group that only will react with another specific reactive group without any potential for cross-reactivity with biomolecule functionalities. In other words, a bioorthogonal reactive group could be added to a complex mixture of biological molecules in aqueous solution without reacting with any of them. Moreover, the ideal bioorthogonal system should be immune to instability in aqueous solutions, such as the tendency to hydrolyze or easily oxidize. True bioorthogonal reagents of this type will link to each other and only each other in the presence of intracellular environments or in cell lysates or in defined biomolecule solutions.

The reality is that there are few options in this category of bioconjugate reagents and each of the systems or reactant pairs that have been developed for bioorthogonal applications have differing degrees of how well they perform in this task. The following sections describe the major chemoselective ligation reactions, which can be considered to have a degree of bioorthogonal characteristics. In each system, the chemoselective pair of reactants can be separately linked or built into the design of crosslinkers or labeling reagents and used to modify biomolecules,

surfaces, particles, or organic compounds. Subsequently, these labeled components can be brought together, even in complex solutions, to facilitate conjugation between the two bioorthogonal reacting species.

In addition, for several reactant strategies in chemoselective ligation, one of the reagent pairs can be designed into a biological monomer that can be utilized by cellular processes to become incorporated into biopolymers. This advantage provides a unique *in vivo* labeling capability through the feeding of monomer analogs to cells or organisms, such as modified amino acids or sugar derivatives, which then get selectively added into proteins, carbohydrates, or lipids. Thus, the ultimate application of bioorthogonal chemoselective ligation is to label specifically the molecules of life directly within living systems and without cross-reactions with other biological functional groups. For a review on the use of chemoselective reactions in living systems, see Prescher and Bertozzi, (2005).

1. Diels–Alder Reagent Pairs

The Diels–Alder reaction has long been a staple for forming carbon–carbon bonds in organic synthesis (Smith and March, 2007). The typical reaction proceeds through the 2 + 4 cycloaddition of a double bond (alkene) and a diene to give a 6-member ring product. The double bond reactant is often called a dienophile, and electron-withdrawing constituents next to the alkene are used to accelerate the reaction (such as COOH, CHO, and COR groups, among others). Conversely, electron-donating groups on the diene are important for increasing reaction rates (Figure 17.1).

Some reports indicated that the Diels–Alder reaction could be done in aqueous environments with a potential for accelerated reaction rates under the right conditions (Rideout and Breslow, 1980; Blokzijl and Engberts, 1992; Pai and Smith, 1995; Otto *et al*., 1996; Wijnen and Engberts, 1997), and the addition of $InCl_3$ was determined to act as a catalyst in aqueous environments (Loh *et al*., 1996). For a review of organic reactions that can be done in aqueous media, see Li (2005).

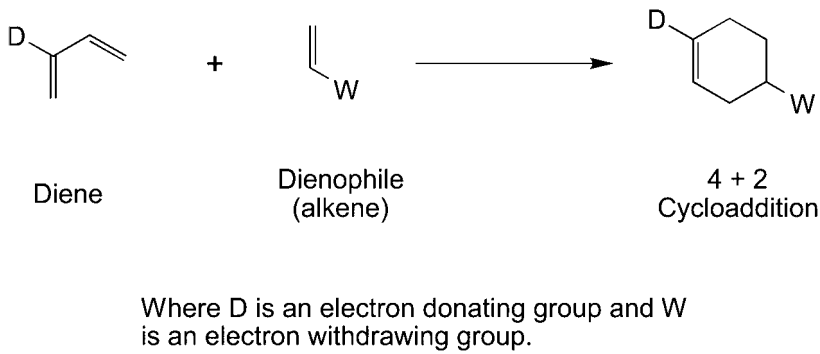


Figure 17.1 A general Diels–Alder reaction consists of a 4 + 2 cycloaddition between a diene and an alkene, often called a dienophile. The reaction rate and yield increase if the diene contains an electron-donating group and the alkene contains an electron-withdrawing group.

In addition, it has been discovered that there are naturally occurring enzymes that facilitate Diels–Alder type reactions within certain metabolic pathways and that enzymes are also instrumental in forming polyketides, isoprenoids, phenylpropanoids, and alkaloids (de Araujo *et al.*, 2006). Agresti *et al.* (2005) identified ribozymes from RNA oligo libraries that catalyzed multiple-turnover Diels–Alder cycloaddition reactions.

In 2001, Hill *et al.* extended the use of aqueous phase Diels–Alder reactions for the bioconjugation of diene-modified oligonucleotides. The dienophile that was used consisted of a simple maleimide derivative, which is present on a broad range of commercially available bioconjugation reagents. Modified oligonucleotides were prepared by solid phase synthesis using a 3,5-hexadiene phosphoramidite derivative, which could be incorporated into the oligo at the *5′* end. Maleimide compounds investigated for oligo bioconjugation include *N*-ethylmaleimide, biotin–BMCC, fluorescein–maleimide, coumarin–maleimide, maleimide–PEG_2–biotin, and an mPEG–maleimide. Figure 17.2 shows the reaction of maleimide–PEG_2–biotin with the diene-modified oligo to yield the cycloaddition product.

The reaction kinetics between a maleimide derivative and a 3,5-hexadiene derivative varies depending on the maleimide compound being reacted. Cycloaddition yields of greater than 80 percent and often as much as 90–95 percent can be expected within 1–18 hours at room temperature or slightly elevated reaction conditions (e.g., 30°C).

The conjugation of oligonucleotides with peptides also can be done using Diels–Alder cycloadditions in water (Tona and Häner, 2005). Marchan *et al.* (2006) used the same 3,5-hexadiene phosphoramidite derivative as Hill *et al.* (2001), but in this case used a maleimide-modified

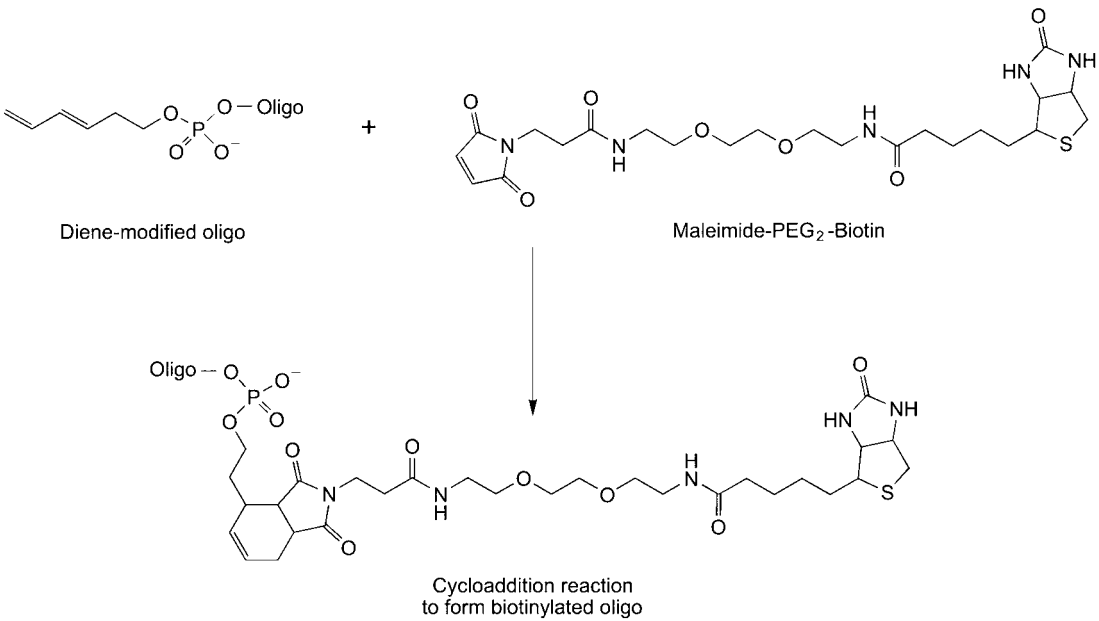


Figure 17.2 Maleimide groups provide good dienophiles for a Diels–Alder reaction. Biotin–PEG_2–maleimide can react with an oligo-diene molecule to form a covalent cycloaddition product, which adds the biotin tag to the oligo.

peptide sequence. A mild, aqueous Diels–Alder reaction between them resulted in the formation of the cycloaddition product.

The Diels–Alder reaction for bioconjugation also has been used for the chemoselective ligation of peptides and proteins in aqueous solution (de Araujo *et al*., 2006). Peptides modified using a 2,4-hexadienyl ester were derivatized to contain a diene and were found to be reactive toward other peptides containing an N-terminal maleimide group to give the cycloaddition product in high yield. The hexadienyl group also was attached to biotin and allowed to interact with streptavidin, which then could be conjugated with peptides containing a maleimide group.

Diels–Alder cycloaddition reactions also have been used to link covalently carbohydrates to proteins (Pozsgay *et al*., 2002) as well as for the immobilization of oligonucleotides on glass surfaces to create arrays (Latham-Timmons *et al*., 2003). In an application that used two chemoselective ligation reactions, Sun *et al*. (2006) employed sequential Diels–Alder and azide–alkyne (click chemistry) cycloaddition reactions to immobilize protein, biotin, or carbohydrate ligands on solid surfaces. In this case, glass slides containing maleimidocaproyl groups were used as the dienophiles and a PEG_4 spacer containing an alkyne on one end and a cyclopentadiene at the other end was the reactive linker. A Diels–Alder reaction coupled the maleimide groups to the cyclopentadiene groups on the spacer, while the alkyne groups at the other end were used in a click chemistry reaction to attach azide-containing ligands (Figure 17.3). The cycloaddition reaction between the maleimide groups on the slides and the cyclopentadiene group on the spacer was done in a 1:1 solution of water:*tert*-BuOH at room temperature for 12 hours. After washing with the same water/solvent mixture, the slides contained hydrophilic spacers terminating in alkyne groups, which then could be coupled with the azide-containing ligands using click chemistry (see Section 4, this chapter).

Chemoselective ligation reactions using the Diels–Alder cycloaddition process offer another bioconjugation route using a reactive component available commercially (maleimide-containing reagents). However, their use as true bioorthogonal reactants is limited due to the cross-reactivity of maleimides toward thiols. For instance, in the modification of a Rab protein, a cysteine residue had to be protected prior to cycloaddition using a maleimide compound (de Araujo *et al*., 2006); otherwise, the maleimide would have coupled to the sulfhydryl, too.

2. Hydrazine–Aldehyde Reagent Pairs

The reaction between an aldehyde or ketone and a hydrazide or hydrazine derivative to form a hydrazone bond has been frequently used for bioconjugation purposes (Chapter 2, Section 5.1). The reaction is appealing from a bioorthogonal perspective, because natural biopolymers don't normally contain these reactive groups. Although aldehydes may form temporary Schiff base interactions with amines on proteins and other biomolecules, in aqueous solution they are fully reversible and will rapidly exchange with a hydrazide or hydrazine, if present. This also holds true of aminoxy (hydroxylamine) derivatives, which form stable oxime bonds with aldehydes, although the number of reagents available with an aminoxy functionality is limited.

The only potential problem of cross-reactivity for this chemoselective reaction pair with molecules of biological origin might occur from a hydrazine reacting with aldehyde or ketone containing metabolic intermediates, reducing sugars, or similar small organic molecules present within cells or cell lysates. However, if the hydrazine reagent is added in sufficient excess, the desired coupling reaction still will occur in such environments, as evidenced by the many

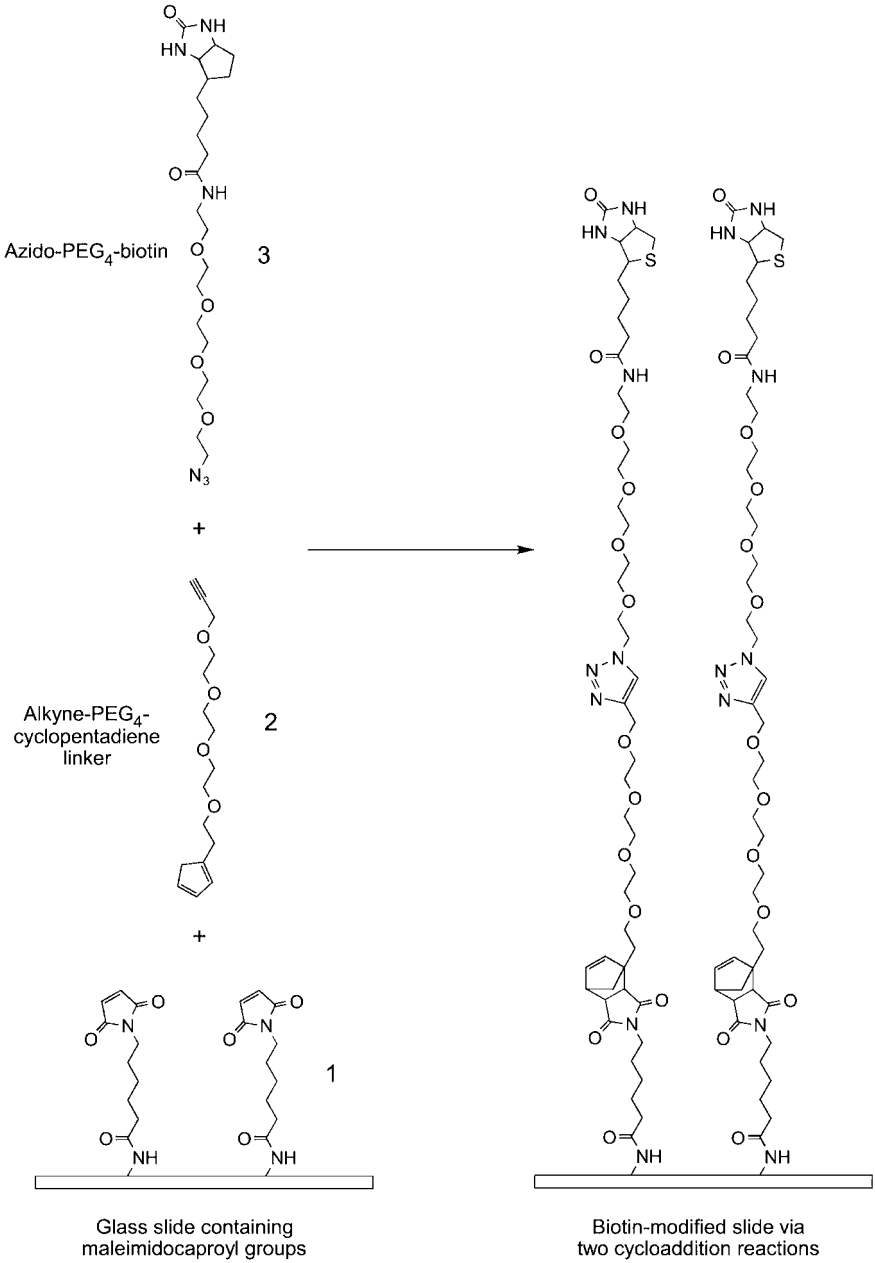


Figure 17.3 Maleimide-modified glass slides (1) can be derivatized using two chemoselective ligation reactions to create biotin modifications. In the first step, alkyne–PEG_4–cyclopentadiene linkers (2) are added to the maleimide groups using a Diels–Alder reaction. In the second reaction, an azido-PEG_4–biotin compound (3) is reacted with the terminal alkyne on the slide using click chemistry to result in another cycloaddition product, a triazole ring.

successful bioconjugation reactions done with oxidized glycoproteins on cell surfaces or in lysates (Bayer *et al.*, 1987a, b, 1990; Bayer and Wilchek, 1990).

The hydrazine–aldehyde reaction has been used intracellularly to deliver non-toxic drug components, which when linked to form a hydrazone bond *in situ*, become cytotoxic (Rideout, 1986, 1994; Rideout *et al.*, 1990). This same approach has been used to generate enzyme inhibitors *in vivo*, wherein the hydrazine and aldehyde precursors are not active, but when coupled together within cells to form a hydrazone linkage, become active site binders (Rotenberg *et al.*, 1991).

Ketone containing bio-monomer analogs have been used successfully to incorporate these functionalities into biopolymers, such as proteins and carbohydrates (glycans). For example, a phenylalanine analog containing a para-substituted aryl ketone group is able to be transformed into an aminoacyl t-RNA and then introduced with sequence specificity into a protein by cellular ribosomal machinery (Datta *et al.*, 2002). In addition, the post-translational glycosylation process within cells is tolerant of certain sugar analogs, and ketone derivatives of monosaccharides have been used to incorporate these functions into glycans. In particular, growing cells in the presence of *N*-levulinoyl-D-mannosamine (ManLev; Figure 17.4) was found to result in the incorporation of this ketone sugar into cell-surface carbohydrates through enzymatic transformation into a keto sialic acid (Mahal *et al.*, 1997; Lemieux and Bertozzi, 1999). Since sialic acid residues are terminal sugars on most mammalian cell-surface glycoproteins, the addition of pendent ketone groups allows for specific targeting of these glycans with hydrazine (hydrazide/aminoxy) based reagents.

There are many bioconjugate reagents and probes available with aldehyde or hydrazide functional groups that may be used for hydrazone bond formation. However, the best choices for making this reaction as chemoselective and bioorthogonal as possible are aromatic aldehyde and aromatic hydrazine derivatives due to their extremely low reactivity with biological functional groups, minimal nonspecific interactions with biomolecules (charge or Schiff base formation), and their highly efficient hydrazone bond formation in complex aqueous solutions (Solulink, Thermo Fisher). Reagents of this type typically contain reactive groups consisting of either an aryl aldehyde (benzaldehyde) or a 6-hydrazinium nicotinate (Schwartz *et al.*, 1993, 1995).

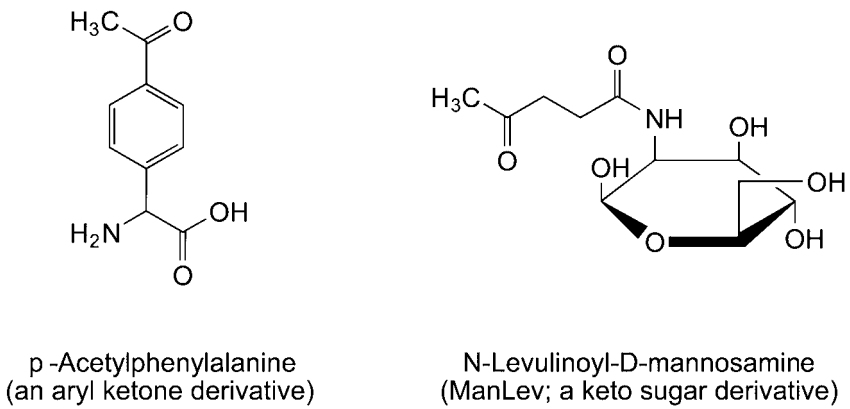


Figure 17.4 Ketone derivatives of phenylalanine and mannose can be fed to cells to incorporate the monomers into proteins and glycans. The resultant modifications can be probed using hydrazide-containing reagents.

These aldehyde and hydrazine functionalities can be associated with virtually any other reactive group or tag, such as heterobifunctional crosslinkers, fluorescent labels, or biotin compounds. Two reagents frequently used to chemically introduce the aldehyde and hydrazine groups include SANH (succinimidyl 4-hydrazinonicotinate acetone hydrazone) and SFB (succinimidyl 4-formylbenzoate) (Figure 17.5). Long-chain analogs of these two compounds also exist, which contain either a 6-aminocaproyl spacer arm in the cross-bridge or a short PEG spacer to promote increased water solubility. The SFB and SANH-NHS ester compounds are amine-reactive and form amide bonds with the molecules modified by them (Figure 17.6). Other reactive groups or tags also are available having the benzaldehyde or hydrazinium nicotinate groups, including maleimide (thiol-reactive), biotin, and various fluorescent probes (Solulink).

The hydrazinium nicotinate group on these reagents commonly is protected against reaction with the active ester by the addition of acetone to form the acetone hydrazone derivative. This hydrazone protective group is readily reversible at neutral or mildly acidic pH and will immediately exchange with a benzaldehyde on the corresponding chemoselective partner to form a stable hydrazone linkage.

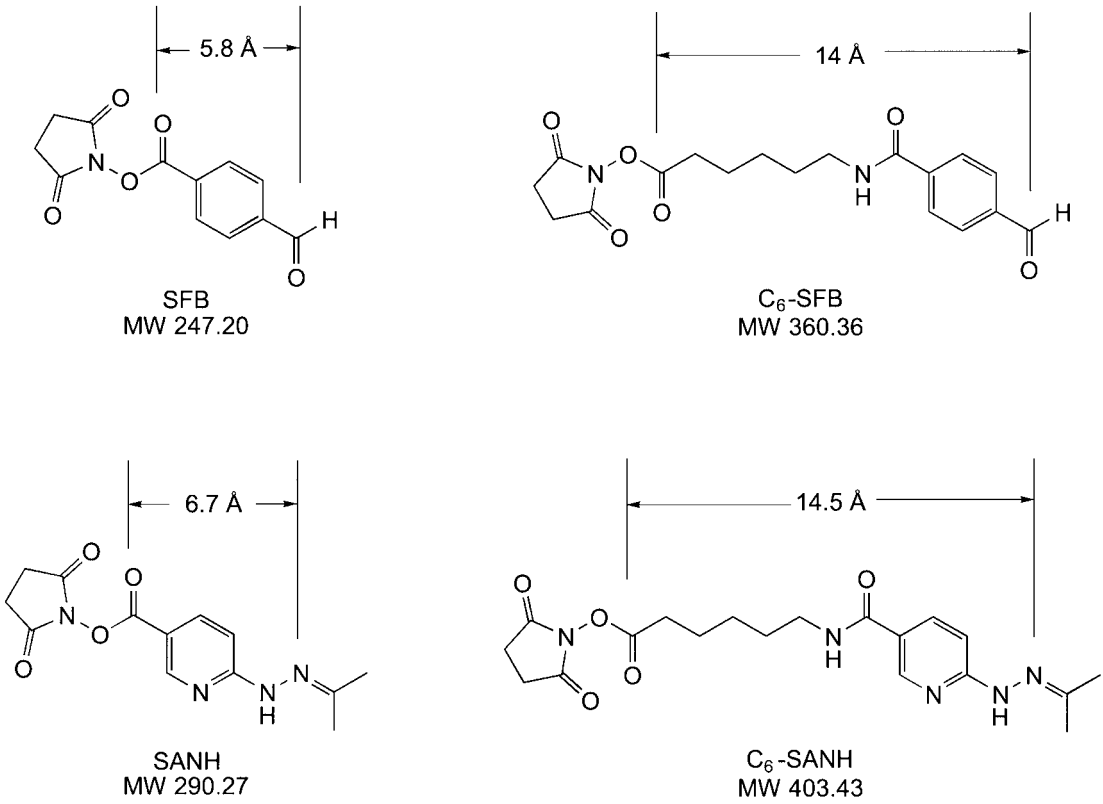


Figure 17.5 Structures of aldehyde-containing and hydrazine-containing heterobifunctional crosslinkers that can be used in chemoselective hydrazone conjugation procedures.

Proteins, other molecules, or surfaces that are activated to contain either the benzaldehyde functionality or the hydrazinium nicotinate group can be quantified as to the number of these groups present using certain chromophoric reactants. For instance, 2-hydrazinopyridine can be reacted with a benzaldehyde-containing molecule to give a UV absorbing derivative with $\lambda_{max} = 350\,nm$ and an extinction coefficient of $18{,}000/cm^{-1}M^{-1}$. Conversely, the hydrazinium nicotinate-activated molecules can be reacted with *p*-nitrobenzaldehyde to create another UV detectable derivative having $\lambda_{max} = 390\,nm$ with an extinction coefficient of $24{,}000\,cm^{-1}M^{-1}$.

The aromatic nature of the SFB and SANH reactants create a hydrazone structure with unique absorptivity for monitoring the course of the ligation process. The aromatic hydrazone bond has a maximal absorbance at 354 nm and a molar extinction coefficient equal to

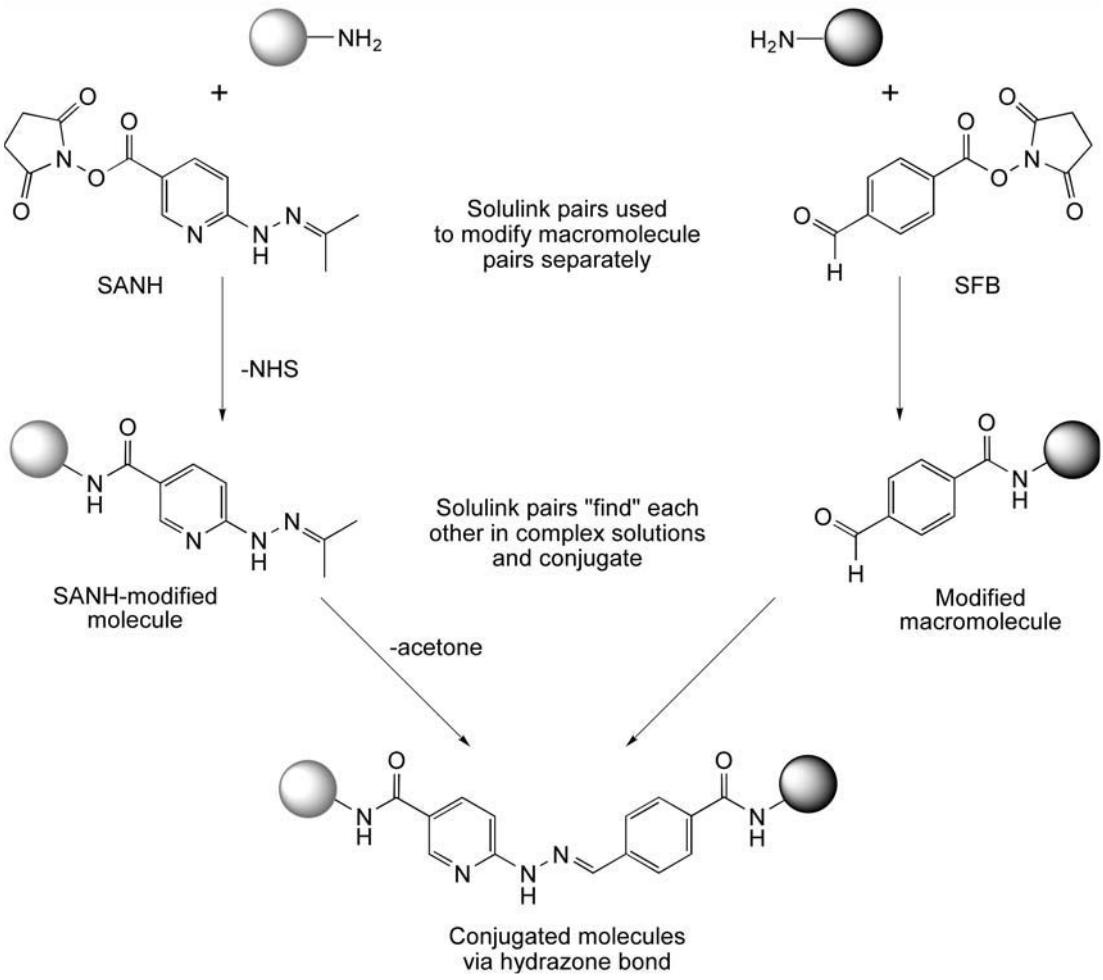


Figure 17.6 The reaction of SANH with amine-containing proteins or other molecules results in amide bond modifications containing terminal hydrazine groups. The reaction of SFB with amine-containing proteins or other molecules results in amide bond modifications containing terminal aldehyde groups. Subsequently, the two modified molecules can be reacted together to create a conjugate via hydrazone bond formation.

29,000/M. If the conjugation reaction is done with sufficient amounts of benzaldehyde and hydrazinium nicotinate reactants, then the yield of the reaction can be directly measured by monitoring the change in absorbance at this wavelength over time.

The bis-aryl hydrazone bond formed by this reaction is stable in aqueous solution over a broad pH range (pH 2–11) and under elevated temperature conditions (up to 94°C) (Solulink web site).

Kozlov *et al.* (2004) used SANH and SFB to create DNA-antibody conjugates for highly sensitive detection in immunoassays using PCR amplification with fluorescently labeled primers. They also investigated hydrazone bond formation and its stability in aqueous solution using 3′ and 5′ labeled DNA pairs, wherein one oligonucleotide contained a benzaldehyde group and the other contained the hydrazinium nicotinate group. It was found that the most efficient pH for the creation of the hydrazone bond was pH 5.0–7.0, with an optimum at about pH 6 and yields dropping off at lower or higher pH. In addition, once the hydrazone bond was formed and the conjugate purified, it was stable in PBS buffer, pH 7.2, with or without 10 mg/ml BSA for 12 weeks at 4°C. The hydrazone linkage also was found to be stable at pH values between 2.3 and 11.3 without hydrolytic breakdown.

When reactions between the benzaldehyde and hydrazinium nicotinate groups are done in relatively dilute solution, for example using a modified antibody molecule at 1 mg/ml (~6 μM) containing one of the two reactants, then to obtain maximal yield of the hydrazone conjugate, the second component should be added in sufficient excess to drive the reaction to completion. Kozlov *et al.* (2004) found that at least an 8-fold molar excess or above of the second reactant over the first reactant is necessary to obtain a yield of about 80 percent hydrazone bond formation.

The practical use of chemoselective ligation reactions for bioconjugation purposes involves the attachment of one of the reactants to a first biomolecule using a standard coupling chemistry (e.g., NHS ester) to an available functional group, while the second reactant is coupled to a second molecule or a surface, depending on the final conjugate desired. In this regard, the following protocols for modification of oligonucleotides or proteins can be done with either the benzaldehyde component or the hydrazine component and the opposite reactant is used to modify another molecule or surface that will be coupled in the final conjugation step.

Protocol for Modification of Amine-Oligo with SANH or SFB

1. Dissolve a 5′-amine modified oligonucleotide at a concentration of 0.5 mM in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4.
2. Dissolve SANH (or SFB) in DMF at a concentration of 100 mM. Note: both reagents are soluble in this solvent to about 50 mg/ml concentration.
3. Add a quantity of SANH or SFB solution to the oligo to provide a 40-fold molar excess of crosslinker over the amount of amine-oligo present. Mix well.
4. React at room temperature for 2 hours to overnight with gentle mixing.
5. Purify the modified oligo from unreacted crosslinker and reaction byproducts using a micro-spin concentrator with a membrane having a molecular weight cutoff able to retain the size of the DNA being modified.

A similar method can be used to modify proteins, such as antibodies, to contain either benzaldehyde or hydrazinium nicotinate groups for subsequent conjugation with another molecule modified by the opposite functionality. The following protocol is based on the methods of Thermo Fisher, Solulink, and Kozlov *et al.* (2004).

Protocol for Modification of Protein or Antibody with SANH or SFB

1. Dissolve the protein or antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4. The antibody solution should be as concentrated as possible, given the amount of antibody available for modification. This general protocol will work for protein or antibody concentrations ranging from about 0.5 mg/ml to 10 mg/ml, but an increase in the molar excess of either SANH or SFB may have to be done at lower antibody concentrations to provide the same modification yields obtained at higher concentrations.
2. Dissolve SANH or SFB in DMF at a concentration of 2 mg/100 µl DMF.
3. Add a quantity of the crosslinker solution of choice (SANH or SFB) to the antibody solution to obtain the desired molar excess of reagent over the antibody. Typically, antibody modification procedures are done with 10- to 20-fold molar excess, but for dilute antibody concentrations, this may have to be doubled, depending on how many hydrazine or aldehyde groups are desired to be introduced on the modified antibody.
4. React for 2–3 hours at room temperature with gentle mixing.
5. Purify the modified antibody by use of size exclusion chromatography or dialysis, using a molecular weight exclusion limit of 5,000–10,000 Daltons. The modified antibody may be stored at 4°C or frozen until used to make a conjugate. The protected hydrazine on SANH is stable for several months and the SFB benzaldehyde group is stable indefinitely. For longer storage, the modified antibody can be lyophilized.

Once a molecule is modified with a hydrazine reagent and another molecule is modified with the benzaldehyde compound, they may be combined to form the final conjugate, which will result in a hydrazone linkage between the two molecules. In addition, chemoselective ligation using aldehyde/hydrazine reactions may be done to immobilize biomolecules. In this regard, one modified component may be a surface and the other one an antibody, protein, or oligonucleotide destined for immobilization onto the surface.

Conjugation Using the Aldehyde/Hydrazine Reaction

1. Separately dissolve the SFB-modified molecule and the SANH-modified molecule in citrate buffer (100 mM sodium citrate, 150 mM NaCl, pH 6.0) at concentrations of at least 1 mg/ml. Note that pH 6 is an optimal pH for the formation of the hydrazone bond, but pH values slightly lower (to pH 4.7) or higher (to pH 7.4) also may work, but they will result in lower reaction rates and lower yields.
2. Mix a portion of the SFB-modified molecule with the SANH-modified molecule to obtain a molar ratio that will give the desired conjugate properties. The optimal ratio of reactants may have to be determined experimentally by performing a series of conjugations using different molar ratios and testing the performance of the final conjugate in the intended application.
3. React for at least 2 hours at room temperature, longer if pH values other than pH 6 are used.
4. To purify the conjugate from reactants that did get incorporated into the conjugate, size exclusion chromatography may be used with resins having a molecular exclusion limit able to accommodate both the labeled molecules and the final conjugate.

3. Boronic Acid–Salicylhydroxamate Reagent Pairs

Phenylboronic acid (PBA) groups can interact with a variety of polar constituents on adjacent or nearby carbon atoms to result in a complex consisting of a 5- or 6-member heterocyclic ring. This process has been used for the affinity chromatographic purification of carbohydrates, glycoproteins, RNA, AMP (from cAMP), glycated proteins (such as glycated hemoglobin formed in diabetes; Klenk *et al.*, 1982), and a range of small molecules containing 1,2- or 1,3-diols, 1,2- or 1,3-hydroxy acids, 1,2- or 1,3-hydroxylamines, 1,2- or 1,3-hydroxyamide, 1,2- or 1,3-hydroxyoxime, as well as various sugars containing these species (Weith *et al.*, 1970; Rosenberg and Gilham, 1971; Rosenberg *et al.*, 1972; Pace and Pace, 1980; Singhal *et al.*, 1980). For a review on the use of PBA in affinity separations, see Scouten (1983). In addition, bioconjugate labeling reagents containing a PBA group also have been used as probes of these species in biological molecules, including fluorescent reagents for targeting glycans on cell surfaces (Burnett *et al.*, 1980; O'Shannessy and Quarles, 1987).

The interaction of PBA derivatives with molecular species having the above functional groups occurs optimally in the pH range of 8–9, but it is typically reversible at acid pH or in the presence of a high concentration of competing ligand. However, the heterocyclic boronic acid complex is relatively stable under optimal conditions of formation.

Stolowitz (1997) exploited this interaction potential in the design of a new chemoselective bioconjugation reagent pair consisting of a PBA group on one reagent and a salicylhydroxamic acid (SHA) group on a second reagent. Each reactant of the pair can be used to modify biomolecules, surfaces, or other compounds for subsequent conjugation or immobilization through specific PBA–SHA ring formation (Springer *et al.*, 2002). The major product of this reaction forms a 6-membered ring structure consisting of the PBA's boron atom along with one of its oxygens coordinated with the SHA's hydroxyl oxygen and hydroxamate nitrogen atoms (Figure 17.7). It also is possible that a 5-membered ring structure can form from interaction of the PBA boron with the hydroxamate hydroxyl and carbonyl oxygens on SHA, but this is a minor product as proven by NMR (Stolowitz *et al.*, 2001).

A significant advancement in the PBA reagent was to add another boronic acid group to the phenyl ring and thus allow two cycloaddition products to form from a single complexation. The phenyldiboronic acid (PDBA) group effectively increases the affinity constant of the interaction if reacting with SHA-modified molecules or surfaces that have more than one near-neighbor SHA group available. The resultant formation of two 6-membered rings per conjugation reaction assures that the bond won't hydrolyze even under high or low pH conditions. This is particularly useful for using the reaction to couple proteins or other molecules to surfaces for arrays, because multiple linkages maintain stability of the immobilized molecule without the possibility for leaching off. A single SHA–PBA bond reportedly has an affinity constant in the range of $10^6\,M^{-1}$, which is relatively weak for affinity binding properties. Two such bonds, however effectively raise the avidity to an observed affinity constant of $>10^{10}\,M^{-1}$ (Lonza product information). Note that multiple single PBA groups also can combine with multiple SHA groups when conjugating proteins together or proteins to surfaces and thus increase the effective avidity of the resultant bonding interaction. Springer *et al.* (2003) describes the use of SHA membranes in this process for the immobilization of PDBA-modified molecules, including nucleic acids and proteins.

Complex formation between PBA or PDBA group and the SHA group occurs in a wide range of buffer types from pH 5 to 9, and it can tolerate high salt conditions (to 1.5 M) or the

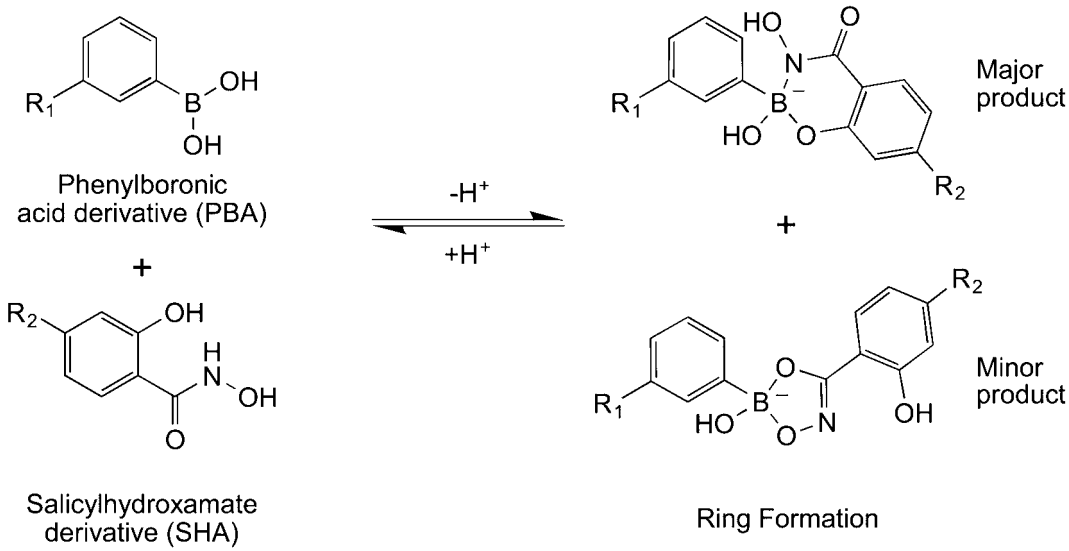


Figure 17.7 PBA derivatives react with salicylhydroxamate derivatives to form 5-membered or 6-membered ring structures, with the 6-membered ring the major product.

presence of moderate amounts of water-miscible solvents, detergents, chaotropes, and denaturing agents commonly used when working with protein solutions.

Ring formation between SHA groups coupled to solid phase matrices and ligands containing either PBA or PDBA groups have been investigated for the immobilization of affinity molecules for protein purification (Wiley *et al.*, 2001). Using the dimeric PDBA group, immobilization of modified alkaline phosphatase onto SHA-agarose resulted in good retention of enzyme activity plus high stability of the coupled protein. The immobilized protein also was stable to elution conditions of acidic (pH 2.5) or basic (pH 11) buffers without cleavage of the boronic acid complexes or leakage of coupled protein. This indicates that multivalent attachment points using PDBA instead of PBA result in high tolerance for extreme environmental conditions without hydrolysis.

Bergseid *et al.* (2000) also reported on the use of SHA-activated chromatography supports for the coupling of boronate-containing affinity ligands. In this case, immobilized RNase A was used to purify anti-RNase antibodies from antiserum samples. RNase A was modified with an NHS–PDBA crosslinker at a molar ratio of 100:1 (crosslinker:protein), purified to remove excess crosslinker, and then coupled to an SHA-agarose support in 0.1 M sodium bicarbonate, pH 8.0.

Prolinx originally developed the technology utilizing the SHA–PBA interaction and later portions of it were commercialized by Invitrogen and then Lonza (Cambrex; Versalinx reagents). A number of PDBA crosslinking agents now are available to introduce the diboronic acid group into biomolecules. These include heterobifunctional compounds containing an NHS ester (amine reactive), a hydrazide group (aldehyde reactive), a maleimide group (thiol reactive), a pyridyl disulfide group for reversible linkage to thiols or for thiolation of target molecules, and an iodoacetyl group for creating thioether bonds. SHA crosslinkers include a hydrazide-containing compound, an NHS ester, and a bis-SHA compound containing a hydrazide group. Some examples of these reagents are shown in Figure 17.8.

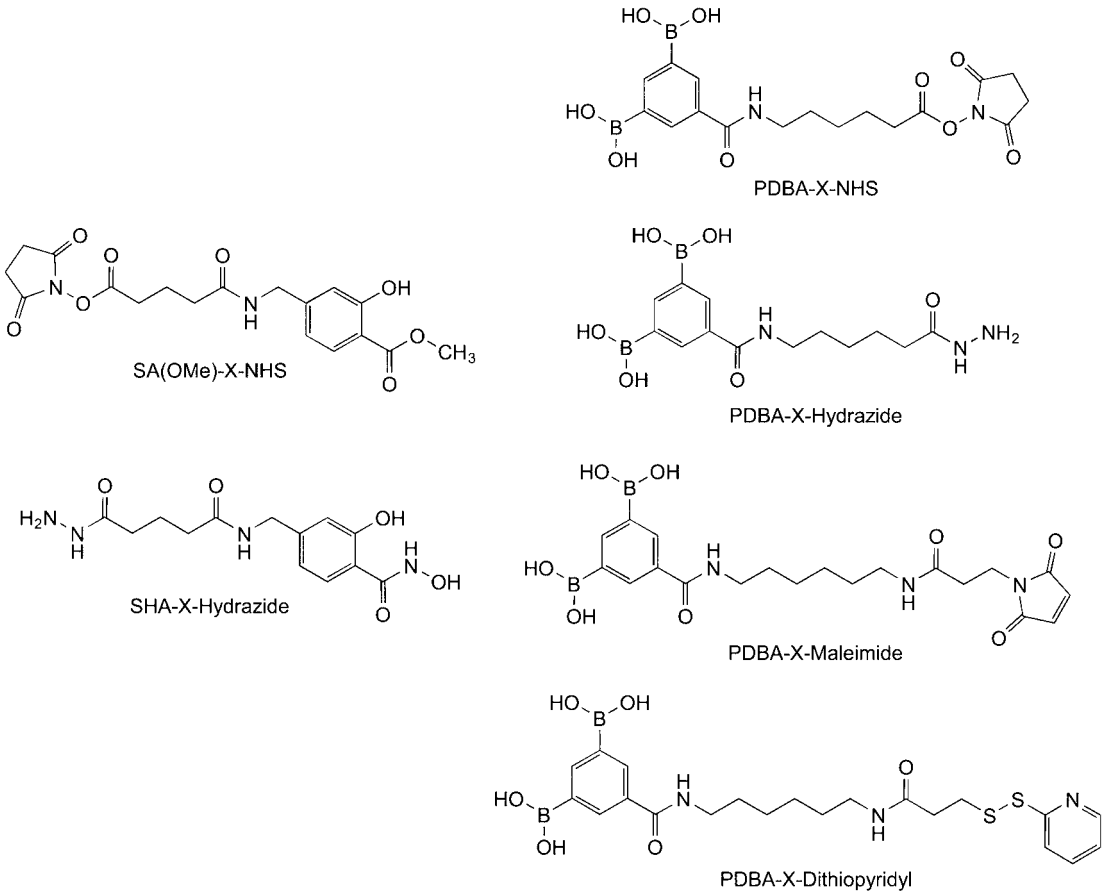


Figure 17.8 Heterobifunctional crosslinking agents containing the PBA or salicylhydroxamate group for chemoselective conjugation purposes.

Conjugation reactions between phenylboronates and salicylhydroxamates entail the formation of a low affinity interaction involving a reversible heterocyclic ring formation. Conjugates formed as a result of this reaction are relatively stable provided the pH is maintained within the optimal range and there are no competing species in solution, which may exchange for the SHA group. This may include sugars or carbohydrates containing diols or other organic constituents mentioned previously. The creation of dimeric or multivalent interactions with two or more SHA groups and a phenylboronate-modified molecule (using PBA or PDBA) will dramatically increase the stability of the linkage over that observed with a single PBA–SHA linkage. Wiley *et al.* (2001) found that modification of alkaline phosphatase with PBA or PDBA for subsequent immobilization on an SHA-agarose support resulted in stable immobilization if 6 PBA groups were present on each alkaline phosphatase molecule or the equivalent of 3 dimeric PDBA groups. This difference reflects the dimeric nature of the PDBA group in forming more than one boronate ring structures per modification.

The use of the PBA–SHA conjugation in a bioorthogonal mode probably will be problematic due to the potential side interactions that could occur involving the phenylboronate groups with biological molecules in cells or cell lysates. In addition, there are no reports of using SHA or PBA bio-monomer analogs (e.g., amino acids containing these groups) to incorporate into biopolymers *in vivo* as there are with other chemoselective reacting groups. However, the use of the PBA–SHA reaction as a chemoselective immobilization process for attaching proteins or other molecules to surfaces may have advantages over other bioconjugation reactive groups, because the reactive groups are very stable and won't hydrolyze in aqueous solution. In addition, the PBA–SHA reaction can be used with success for the conjugation of two pure biomolecules, such as in the creation of a protein–protein conjugate (Le Roch *et al.*, 2000).

The following protocol describes the immobilization of a protein on an amine-surface using the P(D)BA–SHA chemistry.

Protocol

Preparation of P(D)BA-Modified Protein

1. Dissolve a first protein to be modified at a concentration of 1–10 mg/ml in 0.1 M sodium bicarbonate buffer, pH 8.5. PBS buffer at physiological pH also may be used as the reaction medium.
2. Add a quantity of PDBA–NHS to the protein solution to provide the desired molar excess of crosslinker over the protein. A suggested starting point is to use a 10- to 15-fold molar excess of reagent, but the optimal amount to be added should be determined by experimentation to provide a final conjugate having the best possible properties for the intended application. The PDBA-NHS may be first dissolved in DMF as a concentrated stock solution and then an aliquot added to the reaction mixture. Mix well to dissolve.
3. React for at least 1 hour at room temperature with gentle mixing.
4. Purify the modified protein by gel filtration or dialysis using a molecular weight cutoff that will be appropriate for the protein being modified.

Preparation of SHA-Modified Surface

An amine-containing surface, such as an APTS-modified glass slide (see Chapter 13, Section 2), may be modified with a long-chain NHS-salicylic acid methyl ester derivative (Lonza), which then can be converted to the SHA group for coupling to a P(D)BA-modified protein. The following protocol describes this method.

1. Thoroughly wash the amine-slide with D.I. water and then with 0.1 M sodium bicarbonate, pH 10 (coupling buffer).
2. Add a quantity of NHS-salicylic acid methyl ester reagent dissolved in coupling buffer to the slide surface to provide at least a 2-fold molar excess of crosslinker over the quantity of amines present on the surface. The surface of the slide may be coated with a minimum solution volume of the crosslinker by layering the solution over the surface. Slide masks or gaskets may be used to isolate only certain regions for modification. Alternatively, the slide may be immersed in the crosslinker solution. The NHS-salicylic acid methyl ester may be first dissolved in DMF as a concentrated stock solution and then an aliquot

added to the reaction mixture to prepare the final concentration desired. Mix well to dissolve before immediately exposing the solution to the slide surface.
3. React for at least 1 hour at room temperature with gentle mixing.
4. Wash the slide with 10 volumes of water.
5. Prepare a 1 M solution of hydroxylamine in coupling buffer sufficient to again treat the slide. The pH of the coupling buffer should be adjusted to pH 10 after dissolving the hydroxylamine into it. Expose the slide to the hydroxylamine solution in the same manner as the crosslinker treatment. The hydroxylamine will react with the methyl ester groups on the salicylic acids and form hydroxamate functionalities suitable for conjugation with the P(D)BA-modified protein from above.
6. React the slide with the hydroxylamine solution for 16–24 hours at room temperature.
7. Thoroughly wash the slide with coupling buffer and at least 10 volumes of water.

Coupling PDBA-Modified Protein to SHA-Modified Slide

Proteins modified with boronic acid groups may be covalently linked to SHA-modified slides simply by spotting the PDBA-protein onto the slide surface in 0.1 M sodium bicarbonate buffer, pH 8.0. The arraying technique may use pin spotters, piezoelectric contactless printers, or even pipette dispensing into masked wells on the surface. The quantity of PDBA-protein placed on the surface should at least be in 2-fold molar excess to the theoretical density of SHA groups present. Large quantity protein spotting may obviate a covalent attachment strategy by building up a "mountain" of dried protein, as typically is done using simple surface adsorption of protein to form array spots.

4. Click Chemistry: Cu(I)-Promoted Azide–Alkyne [3 + 2] Cycloaddition

The 1,3-dipolar cycloaddition reactions to unsaturated carbon–carbon bonds have been known for quite some time and have become an important part of strategies for organic synthesis of many compounds (Smith and March, 2007). The 1,3-dipolar compounds that participate in this reaction include many of those that can be drawn having charged resonance hybrid structures, such as azides, diazoalkanes, nitriles, azomethine ylides, and aziridines, among others. The heterocyclic ring structures formed as the result of this reaction typically are triazoline, triazole, or pyrrolidine derivatives. In all cases, the product is a 5-membered heterocycle that contains components of both reactants and occurs with a reduction in the total bond unsaturation. In addition, this type of cycloaddition reaction can be done using carbon–carbon double bonds or triple bonds (alkynes).

The reaction between an azide and an alkyne has been referred to as the Huisgen cycloaddition reaction, after the name of its originator (Huisgen *et al.*, 1964). This type of reaction is shown in Figure 17.9 for both alkenes and alkynes, which results in similar heterocycles only differing in a single carbon–carbon double bond within the ring. The Sharpless group has coined the term "click chemistry" to describe these reactions, because of the seemingly "spring-loaded" nature of the electrophiles that participate in it (for review, see Kolb *et al.*, 2001). It also was noticed that such reactions appear to be accelerated in aqueous solution compared to the same reactions done in organic solvent.

Click chemistry reactions historically are done at elevated temperatures, and sometimes elevated pressures, to increase the rate of reaction and make the yield of heterocycle formation acceptable. However, it was discovered that in the presence of Cu(I), the reaction kinetics are

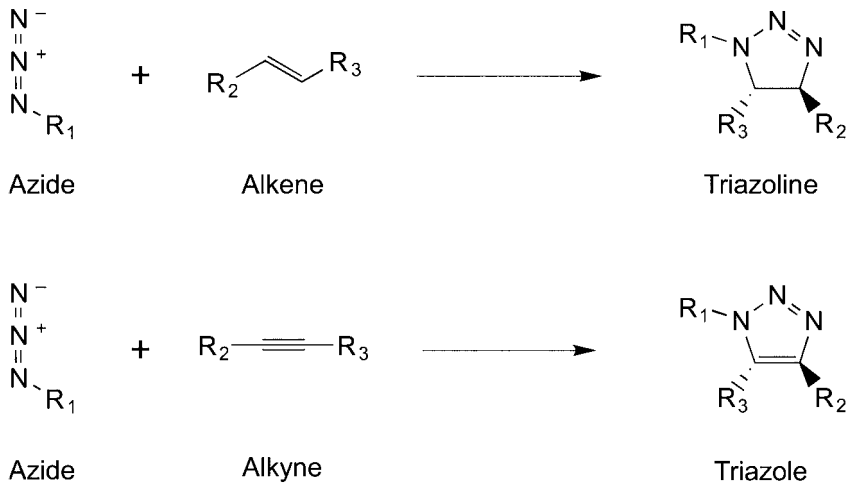


Figure 17.9 A general Huisgen reaction involves the cycloaddition of an azide with an alkene or an azide with an alkyne. The products of these reactions are a triazoline ring or a triazole ring, respectively.

dramatically accelerated to provide high yields even at room temperature and ambient pressures (Rostovtsev *et al.*, 2002; Tornøe *et al.*, 2002; Sharpless *et al.*, 2005). There was an early indication that Cu(I) could catalyze this process (L'abbé, 1984), but it was not pursued further at that time, especially as a potentially useful bioconjugation method. In fact, in aqueous solution, the presence of only a small catalytic amount of Cu(I) in a click chemistry reaction can increase the rate of cycloaddition by about a million-fold, making the reaction biocompatible.

The copper-catalyzed azide–alkyne cycloaddition process has resulted in a proliferation of applications in organic synthesis and bioconjugation. There are hundreds of references to the use of this conjugation reaction for small molecule synthesis, protein conjugation, activity-based protein profiling (ABPP), nucleic acid conjugation, surface modification, detection schemes, and *in vivo* targeting of molecules on cells. One of the primary reasons for the increasing popularity of the click chemistry reaction is the bioorthogonal nature of the two reacting groups.

Azides in particular are convenient electrophilic participants in the click chemistry reaction due to their ease of formation and stability. Alkyl azides undergo nearly no side reactions and are extremely stable in aqueous solution, even in the presence of complex biological material. Note that the aryl azides, which often are used as photoreactive crosslinking agents, are highly unstable to UV light or reducing agents and probably should be avoided for this purpose (although they may be perfectly good substrates for the click reaction).

Alkyne groups also are remarkably stable in biological solutions, provided they don't have an activating group adjacent to them, such as a carbonyl, which would make them predisposed to Michael-type addition reactions, especially with thiols. Adding an alkyne group to a modification reagent or a crosslinker can be as simple as coupling an activated carbonyl group with propargylamine, which forms the propargylamide linkage and creates a terminal acetylene group for conjugation. Link *et al.* (2004) synthesized a biotin–PEG-alkyne modification reagent using this strategy, which then could be used to modify proteins containing azide amino acids (Figure 17.10).

The functional groups used for click chemistry conjugations are completely unreactive toward biological molecules and virtually free of side reactions, which otherwise would cause reagent

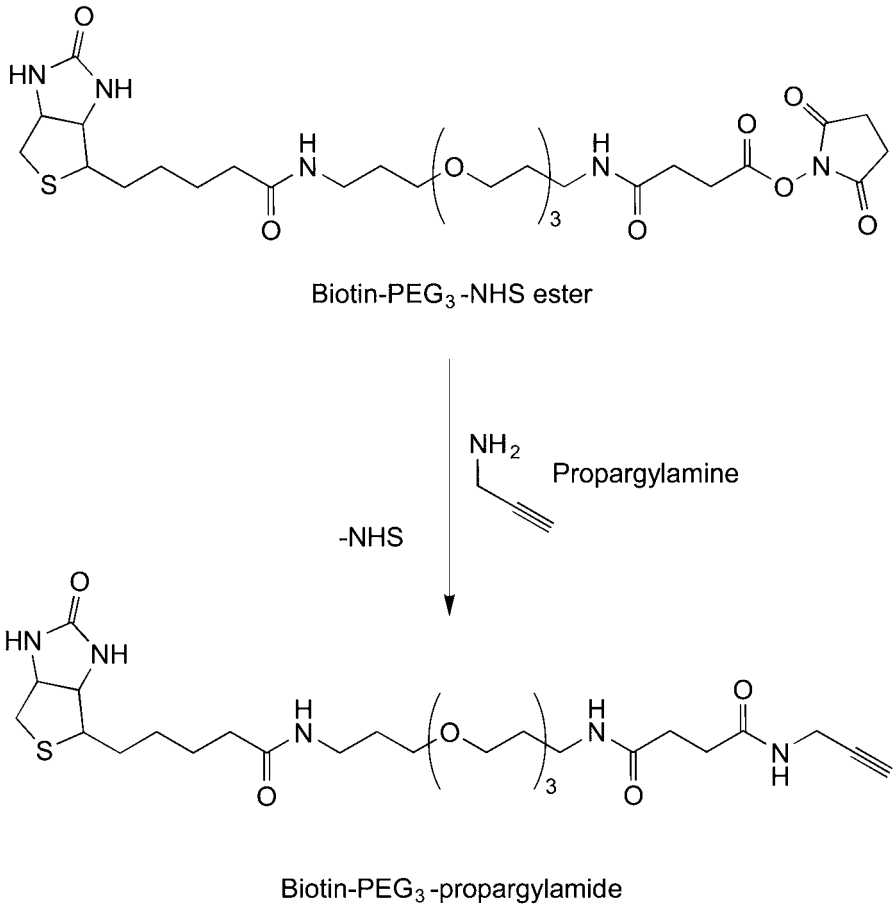


Figure 17.10 Propargylamine can be used to add an alkyne group to amine-reactive reagents, such as the NHS ester group on the biotin–PEG_3 compound.

instability in aqueous environments. This means that a molecule modified to contain an azide functionality would be able to react specifically with another molecule containing an alkyne group, even in the presence of biological fluids, cells, or cell lysates. In addition, without the presence of Cu(I), the azido-molecule and the alkyne-molecule would not react to an appreciable extent at room temperature even when placed together in solution. Only upon the addition of Cu(I) in sufficient concentration would the cycloaddition reaction take place and a triazole linkage be formed.

The Cu(I) source used to drive the click reaction can be generated in several ways. The use of CuBr can be done to add Cu(I) directly to the reaction; however, Cu(I) salts are relatively impure and in solution they are labile and may degrade by oxidation to a significant extent over the time course of the reaction. The use of Cu(I) salts also has been found to result in some degree of side reaction products as well as needing an organic base and organic co-solvent during the reaction to efficiently drive the cycloaddition process. For these reasons, the source for Cu(I) typically is generated *in situ* using Cu(II) in the presence of a reducing agent.

The Cu(II) salt, $CuSO_4$, is particularly convenient, as it is readily available and easily converted to Cu(I) with a reducing agent, such as sodium ascorbate or TCEP. In solution, Cu(II) is reduced to Cu(I) by ascorbate with concomitant oxidation of ascorbate to dehydroascorbate. For reactions between pure click chemistry components in solution, the amount of catalyst addition only has to be from about 0.25 mole percent to 2 mole percent relative to the amount of reactants present, with a 5-fold molar excess of ascorbate over the amount of Cu(II). Therefore the reaction is initiated by production of only a small amount of Cu(I), which catalytically gets oxidized and then regenerated by reduction during the cycloaddition process. The proposed mechanism for the click chemistry reaction has been illustrated as a catalytic cycle by both the Meldal group (Tornøe *et al*., 2002) and the Sharpless group (Rostovtsev *et al*., 2002), giving a cyclic intermediate azide-Cu(I)-alkyne complex, which then goes on to form the 5-membered triazole ring.

Another source for Cu(I) in the click reaction is to use elemental copper metal filings, which generate Cu(I) ion in solution slowly by oxidation. This last option, however, is considerably slower in generating the necessary Cu(I) than the other methods and will result in reactions needing to be done for at least 24 hours.

For click reactions done in complex solutions, such as in the presence of biological molecules, the amount of Cu(II) and ascorbate addition typically is at a concentration of at least 0.1 mM $CuSO_4$ and 0.2 mM ascorbate. In this type of environment, the labeling reaction usually is done on azide or alkyne targets at very low concentration levels and for extended times. At this concentration of metal salt and ascorbate, cells may not remain viable for long periods and may die.

In some cases, click chemistry ligation reactions may not be appropriate for labeling within cells if continued cell viability is important. Live cell labeling requires that the conjugation chemistry not adversely affect cell viability or dramatically alter protein expression or pathway activation. Due to this limitation, the click chemistry reaction has been said to be undesirable for performing conjugations within a living cell, and only useful for labeling targets on live cell surfaces (Link and Tirrell, 2003; Prescher and Bertozzi, 2005).

However, some groups have worked around these issues and developed strategies for live cell labeling wherein the first step occurs *in vivo*, but then subsequent steps use *in vitro* cycloaddition for detection. Speers and Cravatt (2004a, b) used a click chemistry reactant to label enzymes *in vivo* at their active sites with an azide-substrate analog. ABPP typically involves using a binding probe along with a reactive group and a detectable tag, which is able to target specifically the binding site of an enzyme. The reactive group covalently links the affinity molecule to the active site, while the tag is used to image the enzyme *in vitro*. Using the click chemistry strategy, the active site binder in ABPP doesn't contain the detectable tag, but only possesses an azide group. The azide functionality is extremely stable *in vivo*, so the affinity reagent can be used in living cells or whole organisms. After incubation with the azide affinity component, the probe specifically interacts with the enzymes being targeted. Subsequently, the tissue or cells can be lysed (or fixed) and probed for bound enzyme using an alkyne-labeled reagent. This can be a fluorescent probe or an affinity handle, such as biotin, for purification.

In fact, most cell-based assays are done using fixed cells, not live cells, which makes click chemistry reactions imminently practicable. In this approach, a test population and a control population of cells is grown and after treating the test population of cells with a potential drug candidate or another modulator of cellular processes, they are compared relative to the expression of a biological component or the activity of a biomolecule. Most high content screening assays are done on cells after a formaldehyde fixation step followed by a permeabilisation process to allow passage of molecular probes into the cells (refer to Thermo Fisher

Scientific, Cellomics). For these applications, the use of azide/alkyne reagents in a click chemistry strategy is entirely appropriate and may be the best choice of all conjugation reactions, because of its exquisite chemoselectivity, bioorthogonality, and excellent reaction kinetics.

The triazole ring generated by the reaction of an azide and an alkyne is a very stable linkage and not likely to undergo hydrolysis or any other breakdown reaction to cleave the linkage. Even under relatively extreme conditions used in some biological operations involving the addition of denaturants, detergents, chaotropic agents, organic solvents, or acidic or basic conditions, the triazole ring will survive and remain intact. Click chemistry reactions are thus highly chemoselective and result in strong conjugation bonds for use in any application.

Another important advantage to the use of click chemistry for cell-based targeting is the ability to create bio-monomer analogs containing either azido or alkyne functionalities, which then can be incorporated into biopolymers using a cell's native enzymatic machinery. Methionine and phenylalanine analogs containing side chain alkyne or azide groups have been synthesized and proven able to be introduced into proteins at normal methionine sites in a sequence-specific manner (Link *et al.*, 2004; Prescher and Bertozzi, 2005). Kiick *et al.* (2002) also demonstrated that both azido and alkynyl amino acid derivatives could be used as methionine surrogates and get integrated into proteins with nearly the same efficiency as normal methionine. In addition, azide-sugar derivatives have been prepared that are capable of being incorporated into glycans and glycoconjugates using normal enzymatic biosynthetic pathways in cells (Saxon and Bertozzi, 2000). Thus, proteins and carbohydrates can be specifically tagged to contain non-canonical amino acids or sugars for subsequent bioconjugation using reagents containing the opposite click chemistry reactant (Figures 17.11 and 17.12). Cells grown in the presence of azido or

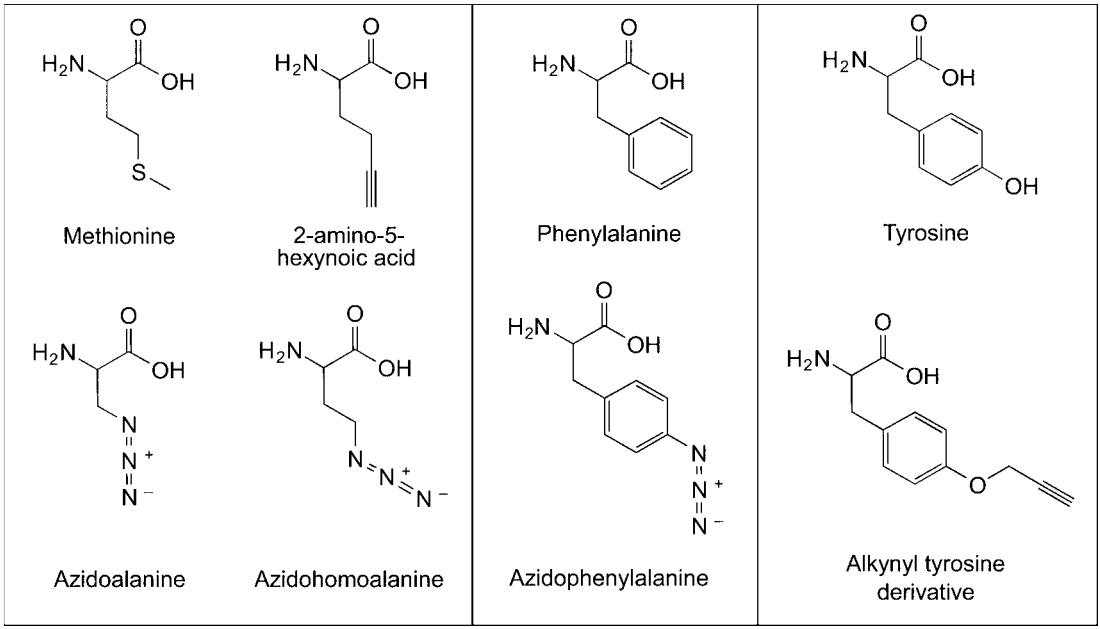
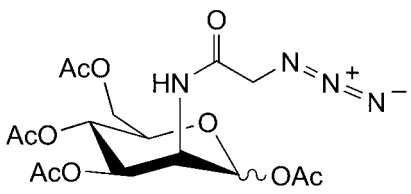


Figure 17.11 Amino acid analogs containing either azido or alkyne modifications can be fed to cells and these monomers incorporated into expressed proteins.

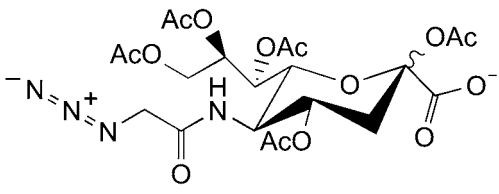
alkyne monomer analogs will utilize these as building blocks for creating biopolymers. Proteins and glycans within the cells and on cell surfaces afterward display discrete bioconjugation targets for subsequent detection, crosslinking, or capture using click chemistry applications.

Lin *et al.* (2006) used click chemistry combined with site-specific labeling of recombinant protein using expressed protein ligation (EPL) (Muir, 2003) to couple proteins to array surfaces. A propargylamido-cysteine reagent was used to modify an expressed protein containing a thioester intein at its C-terminal. Reaction of the free thiol on the propargylamido-cysteine with the thioester linkage on the protein resulted in transthioesterification followed by an immediate S→N shift to give an amide bond between the alkyne compound and the protein (Figure 17.13).

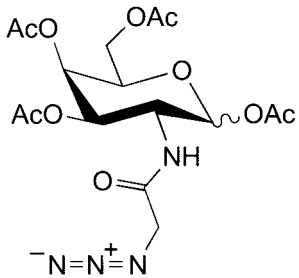
Once the protein is modified to contain an alkynyl group at its C-terminal it can be used to covalently link to its click chemistry reactant partner, an azide on the surface of an array. Other azido molecules also can be conjugated with an alkyne-protein to facilitate the detection or capture of the protein using affinity techniques. For instance, an azido-fluorescein reagent can be used to detect fluorescently the expressed protein in complex samples or an azido-biotin



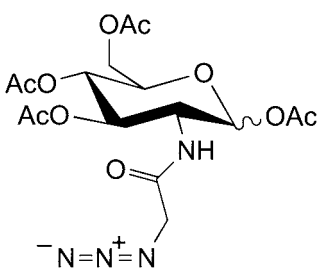
Azido-N-acetyl
mannosamine
derivative (ManNAz)



Azido-N-acetyl
sialic acid derivative
(SiaNAz)



Azido-N-acetyl
galactose derivative
(GalNAz)



Azido-N-acetyl
glucose derivative
(GlcNAz)

Figure 17.12 Azido derivatives of sugars can be used as monomers for glycan and carbohydrate synthesis by cells. Such modifications can be probed using click chemistry or Staudinger ligation reactions.

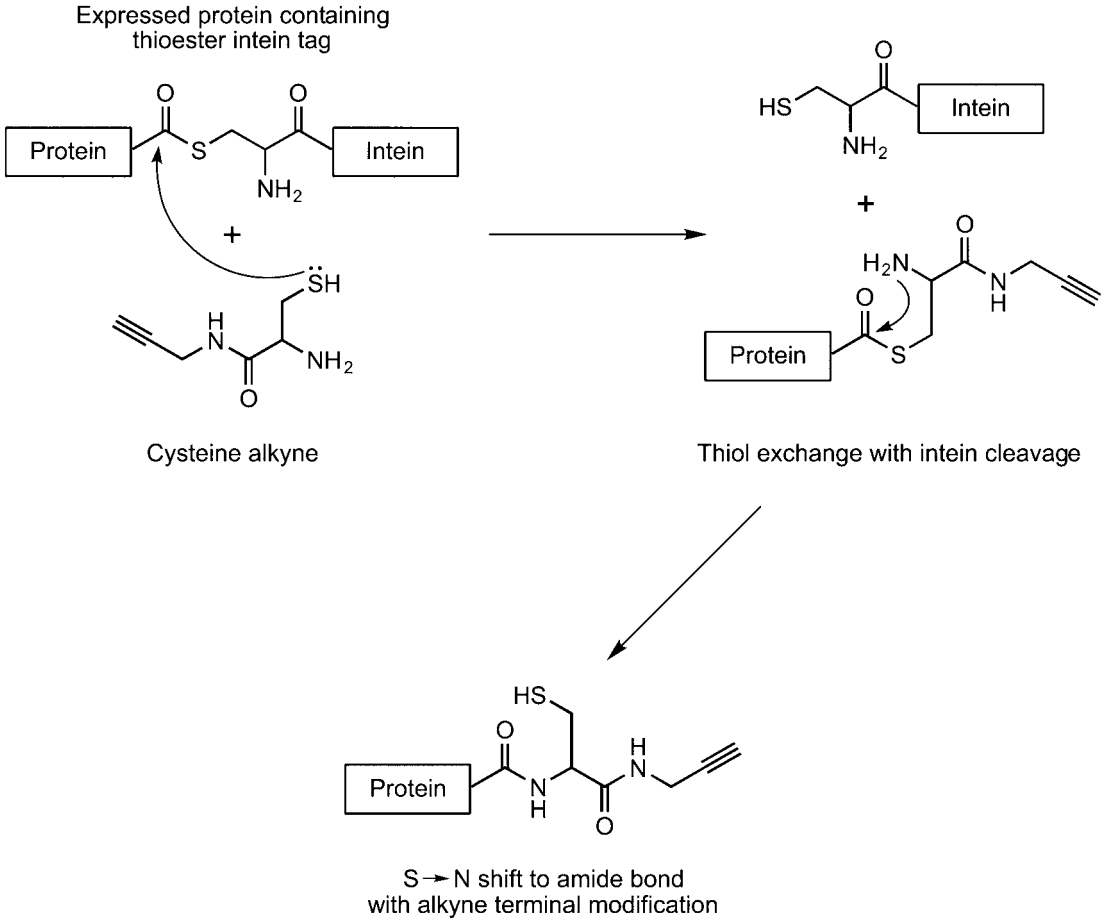


Figure 17.13 Expressed proteins containing a thioester intein tag can be specifically modified using a cysteine-alkyne derivative by transthioesterification followed by an internal S → N shift.

derivative can be used to biotinylate the protein at its C-terminal for subsequent purification or detection (Figure 17.14).

In another application of coupling proteins to surfaces using click chemistry, Duckworth *et al.* (2006) carried out prenylation of a protein using a farnesyl azide derivative and the enzyme farnesyl transferase for subsequent chemoselective ligation to alkyne-functionalized agarose beads. The result is a highly discrete, site-specific attachment of the protein to the solid phase at a single location.

Bonnet *et al.* (2006) used the click chemistry reaction to synthesize receptor ligands containing fluorescent probes or biotin groups to specifically tag and detect or isolate receptor proteins. The efficiency of the click cycloaddition reaction catalyzed by Cu(I) presented many benefits over doing such synthesis by other routes. If receptor ligands can be modified to contain an alkyne group or an azide, then the opposite click chemistry reactant can be used on any number of probes to conjugate with the ligand for subsequent receptor probing applications.

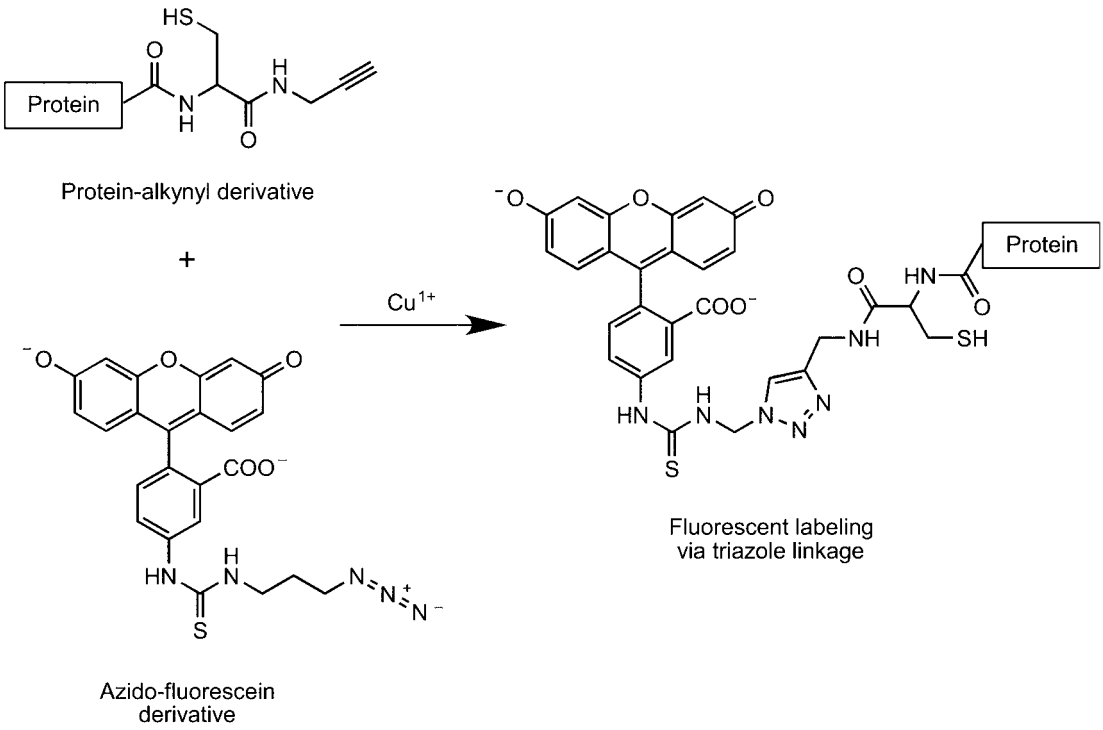


Figure 17.14 An expressed protein containing a thioester intein tag that was subsequently modified by native chemical ligation to contain an alkyne group then can be labeled using an azido-fluorescein probe by the click chemistry reaction in the presence of Cu^{1+}.

Nanoparticles also can be modified with one of the click chemistry reactants to facilitate protein or ligand coupling to them in high yield. Brennan *et al.* (2006) modified gold nanoparticles with a thiol-azido spacer to produce azide functional groups on the particles for subsequent coupling of alkynyl-lipases. The thiol end formed a dative bond with the gold surface, which produced a long, hydrophilic, PEG-containing spacer terminating in an azide (Figure 17.15). Interaction of the alkynyl-lipase with the modified particles in the presence of Cu(I) resulted in triazole linkages, which efficiently immobilized the enzyme on the surface. The resultant enzyme-gold particles retained high enzymatic activity with an average of 7 protein molecules conjugated per nanoparticle.

The following protocol adapted from Brennan *et al.* (2006) describes the coupling of alkyne-modified protein to 14 nm gold nanoparticles. The lipase enzyme was engineered to contain a single lysine residue that was accessible to the aqueous environment for labeling with 4-pentynoic acid using carbodiimide coupling. This procedure also is applicable to other proteins containing lysine residues to add alkynyl groups for subsequent click chemistry conjugation procedures.

An alternative method of modifying proteins to contain alkynyl groups is to use the propargyl–PEG_1–NHS ester compound described in Chapter 18, Section 2. This reagent will react spontaneously with available amine groups in proteins to form an amide bond without the need to use EDC, as in the following protocol.

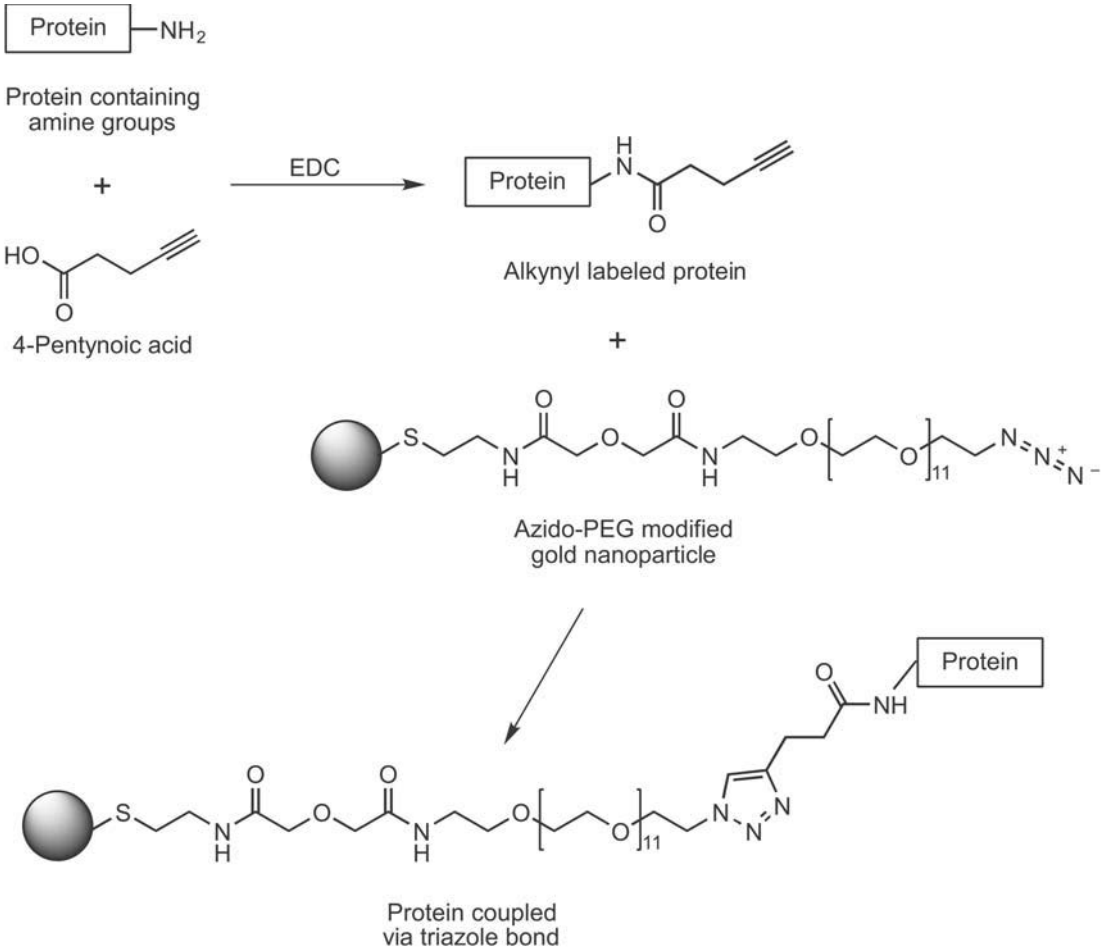


Figure 17.15 The small carboxylate-alkyne compound 4-pentynoic acid can be used to modify proteins at their amine groups with EDC to provide alkyne sites for click chemistry-mediated conjugation. The subsequent reaction of an azido-PEG-modified gold nanoparticle with the alkynyl-protein in the presence of Cu^{1+} yields the triazole-coupled protein.

Protocol

Modification of Protein with Alkynyl Groups

1. Dissolve a protein to be modified with an alkynyl group in 1.2 ml of 20 mM sodium phosphate, pH 7.0 (PBS), at a concentration of at least 20 μM.
2. Prepare a stock solution of 4-pentynoic acid by dissolving it at a concentration of 100 mM in 50 percent THF/PBS. Add 6.7 μl of this solution to the protein solution with mixing.
3. Prepare a stock solution of EDC just prior to use by dissolving it in PBS at a concentration of 50 mM. Note: EDC is unstable in aqueous environments and should be used immediately after the solution is made. Quickly add 13 μl of the EDC solution to the protein solution with mixing.

4. Add to the reaction solution with mixing 150 µl of THF and 130 µl of PBS.
5. React with mixing in a fume hood overnight at room temperature.
   Note: this reaction may be complete within 2–4 hours, as carbodiimide conjugations usually proceed to completion within this time frame.
6. Purify the alkynyl-protein by dialysis or gel filtration using PBS to remove excess reactants and solvent.

Modification of Gold Nanoparticles with Thiol-PEG-Azide Linker

1. Gold nanoparticles (or other metallic or semiconductor particles) are functionalized with azide groups by suspending a 2.8 nM concentration of particles in 20 ml of water and adding a 20 µmole amount of a thiol-PEG-azide spacer ligand to the suspension with stirring.
2. Mix for 18 hours at room temperature.
3. Purify the modified particles from excess linker by repeated centrifugation (at least 3 times at 15,000 g) and resuspending each time with water.

Coupling Alkyne-Protein to Azide-Nanoparticles

1. Suspend the azide-nanoparticles in water at a concentration of 13 nM.
2. With mixing, add a quantity of alkyne-modified protein to the particle suspension to provide at least a 10-fold molar excess over the quantity of azide groups present on the particles. The high molar excess is important to prevent particle aggregation if the modified protein has more than one alkyne group, which could crosslink more than one particle with a single protein, if the protein concentration is too low. However, if the modified protein only has a single alkyne modification, reacting it at a lower molar ratio is okay. For example, Brennan *et al*. (2006) added 36 µl of alkyne-lipase (69 µM) in PBS buffer to 192 µl of azide-nanoparticles (13 nM).
3. Add 2.5 µl of 10 mM $CuSO_4 \cdot 5H_2O$, 50 mM ascorbic acid dissolved in water per ml of the protein and particle mixture. Mix to dissolve.
4. React at room temperature for up to 3 days (or at 4°C, if the protein is not stable at ambient temperature). The optimal time of reaction is dependent on the protein being coupled and the number of alkyne reactive groups available. An alkynyl-protein added to the nanoparticles at a high molar ratio probably would reach maximal coupling yield in a matter of hours.
5. Purify the protein-particles by repeated centrifugation (at least 3 times @ 15,000 g) and resuspending each time with water.

In another application to couple ligands to surfaces, Sun *et al*. (2006) used two chemoselective ligation reactions, a Diels–Alder cycloaddition followed by an azide–alkyne (click chemistry) reaction to immobilize protein, biotin, or carbohydrate ligands on glass slides. In this strategy, glass slides were prepared containing maleimidocaproyl groups, which were used in the Diels–Alder reaction to couple a PEG_4 spacer containing an alkyne on one end and a cyclopentadiene at the other end. The Diels–Alder reaction was used to link the cyclopentadiene to the maleimide groups, while the alkyne groups at the other end were used in the click chemistry reaction to attach the azide-containing ligands (see previous Figure 17.3).

Click chemistry reactant pairs used for surface immobilization have the advantage of being stable to aqueous conditions and long-term storage. Unlike many of the other coupling chemistries

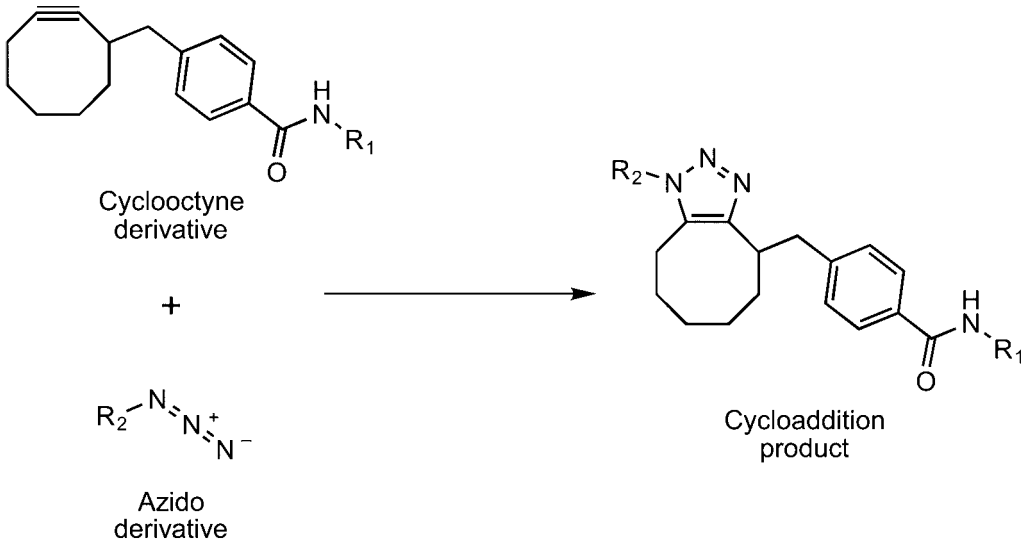


Figure 17.16 Cyclooctyne derivatives can be used as alternative click chemistry reactants, as they are capable of reacting with an azide group without the presence of Cu^{1+} to form a cycloaddition product. This reaction proceeds at a slower rate than the Cu^{1+}-catalyzed process, but it avoids the cytotoxic effects that copper addition can have on cells.

used with surfaces (e.g., NHS esters, EDC conjugation), which suffer from hydrolysis and degradation over time, the alkyne or azide components can be used to activate a surface and stored indefinitely until needed. A ligand modified with the opposite reactant then can be spotted on the array surface in the presence of Cu(I) to initiate covalent attachment through triazole ring formation.

Another version of the click chemistry azide/alkyne reaction has been developed to eliminate the requirement for Cu(I) to be added to catalyze the triazole ring formation. Agard *et al.* (2004) used a cyclooctyne ring to take the place of the typical linear alkynes used as the reactant partner for azides in the Cu(I) catalyzed reactions. This cyclic triple bond reactant is activated, because of ring strain, to produce better kinetics in the 3 + 2 cycloaddition reaction (Prescher and Bertozzi, 2005; Agard *et al.*, 2006). The reaction with an azide lessens the ring strain of the alkyne within the cyclooctyne structure, and thus drives the reaction without the addition of cytotoxic copper (Figure 17.16). This cycloaddition reaction still is much slower than a copper-catalyzed reaction, but its usefulness on living cells may give it an advantage in this application.

5. Staudinger Ligation

Early in the last century, the Nobel Prize winning chemist Hermann Staudinger discovered a reaction between phosphines and azides, which became known as the Staudinger reaction (Staudinger and Meyer, 1919). Triphenylphosphine reacts with azides to form an intermediate iminophosphorane with the release of nitrogen gas. This intermediate quickly breaks down in aqueous environments to yield triphenylphosphine oxide and a primary amine (Figure 17.17).

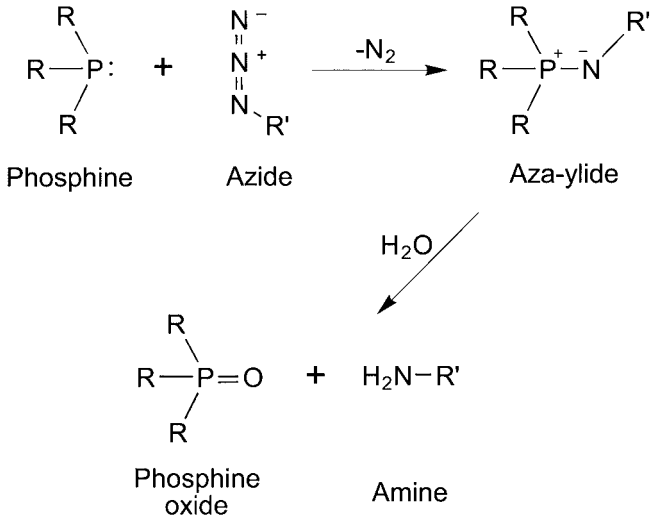


Figure 17.17 The Staudinger reaction involves the reduction of an azide to a primary amine with loss of N_2 and the concomitant oxidation of a phosphine derivative to a phosphine oxide.

This reaction has since been used successfully to synthesize amines in countless numbers of organic compounds and still remains one of the most common organic reactions performed today. Often azides are thought of as hidden amines, because the azide is relatively inert to other reactants until it is revealed through the Staudinger reaction.

A significant modification to the Staudinger reaction was developed by Saxon and Bertozzi (2000) that effectively turns it into a covalent coupling reaction with bioconjugation potential (see also Saxon and Bertozzi, 2003, 2006). Termed "Staudinger ligation", this reaction is done using a triphenylphosphine derivative that contains an electrophilic group next to the phosphorus core. Ortho positioning of an electrophilic benzyl methyl ester group on one of the phenyl rings provides a reactive site for the nucleophilic nitrogen from an azide group that temporarily forms an aza-ylide interaction with the phosphorus atom core. Nucleophilic attack of the nitrogen on the carbonyl "electrophilic trap" releases methanol and forms a stable amide bond (Figure 17.18).

Thus, using the Staudinger ligation process, a triphenylphosphine derivative containing a benzyl methyl ester group can be covalently conjugated to an azide derivative through the formation of an amide linkage. The two derivatives can have attached to them virtually any other molecules, such as proteins, carbohydrates, other biological molecules, fluorescent tags, biotin groups, or other organic compounds, to create a conjugate between them.

The Staudinger ligation reactants also are extremely bioorthogonal. As discussed previously in the section on click chemistry, the (aliphatic) azide component is extremely stable in aqueous or biological solution and won't cross-react with other functional groups. The azide derivative is not appreciably reduced even given the reducing potential inside cells. In addition, the aryl derivative of triphenylphosphine used as a partner in this reaction also has been shown to be very stable in complex solutions, even to the point of not being an effective reducing agent for disulfides, as are many other phosphine compounds, including triphenylphosphine (Saxon and Bertozzi, 2000).

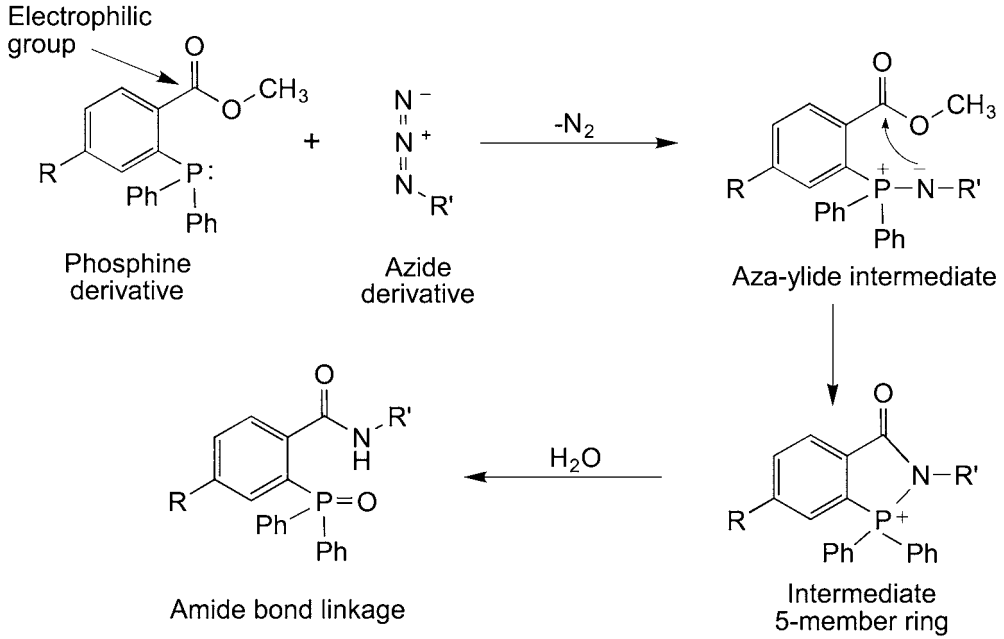


Figure 17.18 The Staudinger ligation reaction uses a modified phosphine derivative containing an electrophilic group that acts as a trap for the nucleophilic nitrogen in the intermediate aza-ylide. The resultant shift yields an amide bond derivative between the phosphine-containing molecule and the azide-containing molecule.

There now are available a number of alkyl azide compounds that may be used in click chemistry reactions and the Staudinger ligation processes. It is not recommended, however, to use aryl azide compounds, as these are light sensitive and photoreactive as well as highly susceptible to reduction in the presence of thiols. Unfortunately, at the time of this writing there are fewer choices in aryl phosphine compounds to participate in this reaction, as commercial sources of labeling reagents are limited.

The reaction between an alkyl azide and the aryl triphenylphosphine occurs without the requirement for other catalysts or activators, such as the need for Cu(I) in the click reaction. This provides an advantage for working with biological samples, since there is no possibility for toxicity or side reactions with added components being present. For this reason, the Staudinger ligation reaction is very amenable to being done with living cells without affecting cell viability.

Additionally, the azide group can be incorporated into amino acids, sugars, and lipids to label cellular molecules *in vivo* prior to reacting with a phosphine probe (see the previous Figures 17.11 and 17.12). Kiick *et al.* (2002) demonstrated that both azido and alkynyl amino acid derivatives could be used as methionine surrogates and get integrated into proteins with nearly the same efficiency as normal methionine. Growing cells in the presence of one of these azido monomers results in their incorporation into biopolymers at specific sites. In this manner, biomolecules can be purposely tagged using Staudinger ligation with a detectable phosphine probe, which is all done within living cells to track or locate them within their native environment. For instance, Prescher *et al.* (2005) used azido-sugar derivatives to modify cell-surface

glycans with azide groups at sialic acid residues, which are typically the outer-most sugars on glycoproteins. It was found that cells most effectively took up these compounds if the hydroxyl groups were acetylated, presumably due to the easy transport of such hydrophobic derivatives through lipid membranes. The acetyl groups are removed within the cells by esterases, thereby providing the azido-sugar to the cell machinery for synthesizing glycoconjugates. The azido-glycan modifications produced on the cell surface using this process then could be labeled by the Staudinger ligation reaction with a phosphine-Flag-tag derivative and subsequently detected using fluorescently labeled anti-Flag tag antibody and flow cytometry.

Due to the bioorthogonal nature of the reactants used for Staudinger ligation as well as the ability to incorporate azido analogs in biopolymers *in vivo* and the mild effect the reagents have on living cells, the process has been termed "a gift to chemical biology" (Köhn and Breinbauer, 2004). For the first time, efficient labeling of biomolecules within cells can be done with very small modifications to the biopolymers *in vivo*. Staudinger ligation permits discrete chemical tagging with detectable probes or affinity handles that facilitate purification.

The methods used for *in vivo* incorporation of azido-monomers and performing a labeling reaction with live cells are relatively simple. The following protocol is based on the methods of Saxon and Bertozzi (2000), which uses acetylated azidoacetylmannosamine as the azido-monomer source and a biotin–PEG–phosphine compound to biotinylate cell surface glycoproteins at the specific azide-sialic acid incorporation sites (Figure 17.19).

Protocol

1. Grow cells ($\sim 1 \times 10^5$ cells/ml) for 3 days in appropriate media containing a 20 μM concentration of acetylated azidoacetylmannosamine.
2. Wash the cells at least twice with 0.1 percent fetal bovine serum in 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4 (PBS) to remove excess azido-sugar.
3. Suspend the washed cells in 0.25 ml of PBS, pH 7.4.
4. Add to the washed cells 60 μl of a 5 mM concentration of the phosphine derivative to couple to the azido-sugar groups on the cell surface (e.g., biotin–PEG–phosphine).
5. Incubate for 1 hour at room temperature with gentle mixing.
6. Wash the cells with PBS, pH 7.4, to remove excess biotinylation reagent.

The biotinylated glycans on the cell surfaces subsequently may be probed with (strept)avidin reagents to detect the azido-sialic acid modifications. Alternatively, the cells may be lysed and the glycoproteins isolated using an immobilized (strept)avidin or monomeric avidin affinity resin.

The same approach to *in vivo* labeling may be done using azide derivatives of sugars administered intraperitoneally in mice (once per day for 7 days) (Prescher *et al.*, 2005). Tissue samples then can be taken of particular organs and reacted *ex vivo* with a phosphine derivative to undergo Staudinger ligation. This process can facilitate probing of glycans on the cell surfaces of organs or cells within an animal. Various treatment procedures can be done, such as administering drug candidates to test animals, to determine the effect on glycosylation of proteins *in vivo*.

Staudinger ligation techniques also can be used to detect post-translational modification of proteins *in vivo*. Hang *et al.* (2007) developed a method to monitor fatty acid acylation of proteins using azido-fatty acids fed to cells. The two major types of fatty acid acylation,

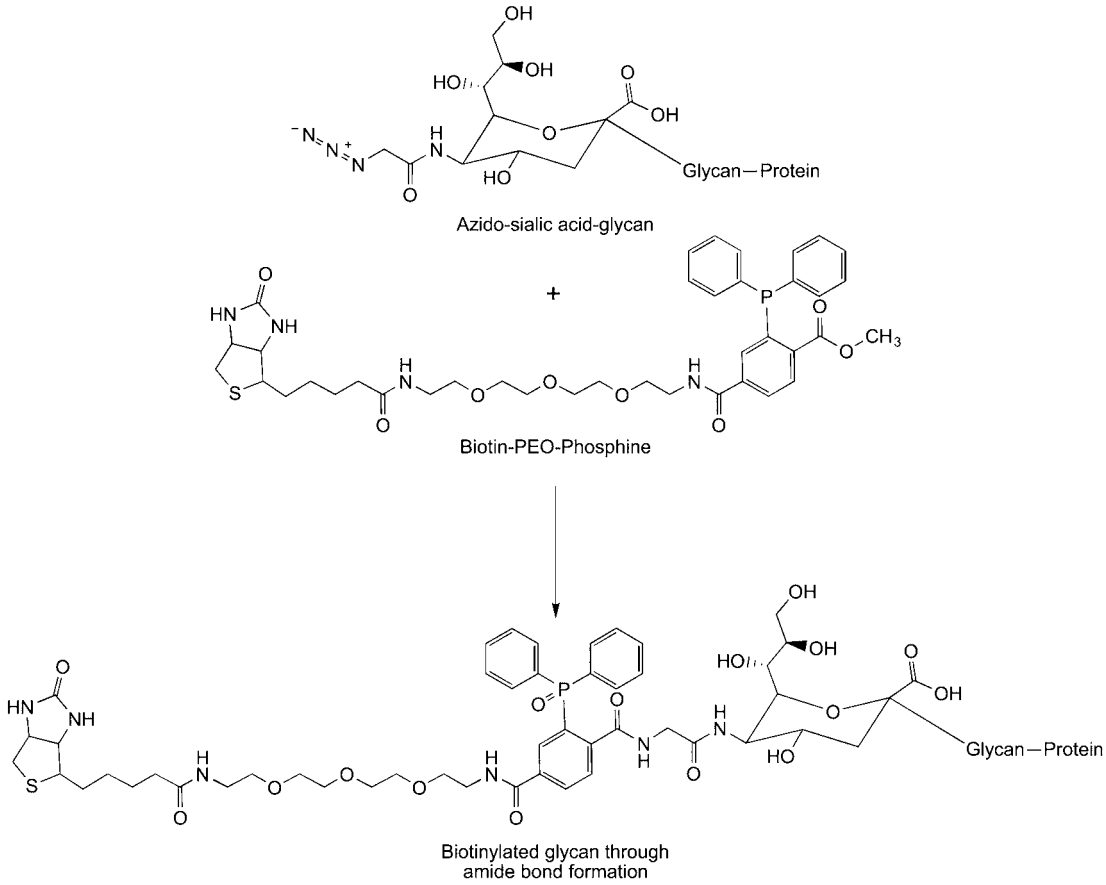


Figure 17.19 An azido-sialic acid derivative that gets incorporated into glycans in cells can be labeled specifically with a biotin-phosphine tag using the Staudinger ligation process. The result is an amide bond linkage with the glycan.

N-myristoylation and *S*-palmitoylation, could be detected with terminal (ω-)azido labeled myristic acid or palmitic acid. Mammalian cells grown in the presence of these fatty acid azide derivatives resulted in certain proteins being modified to contain ω-azido-fatty acid modifications. Subsequent Staudinger ligation with a biotin–PEG–phosphine reagent resulted in covalent attachment to any post-translationally modified protein containing these groups (Figure 17.20). The biotin group then could be used for detection or purification of *N*-myristoylated or *S*-palmitoylated proteins using (strept)avidin reagents.

In a similar application, Kho *et al.* (2004) were able to detect post-translationally modified proteins using an azido-farnesyl analog. Cells grown in the presence of this derivative enzymatically incorporated the azido group into farnesylated proteins through the action of farnesyl transferase. These modifications then could be targeted through Staudinger ligation using the same biotin–PEG–phosphine reagent (Figure 17.21).

Protein farnesyl transferase also can be used to add a geranylazide derivative to a synthetic peptide by incorporating the enzyme recognition sequence "CAAX" at the C-terminal of any

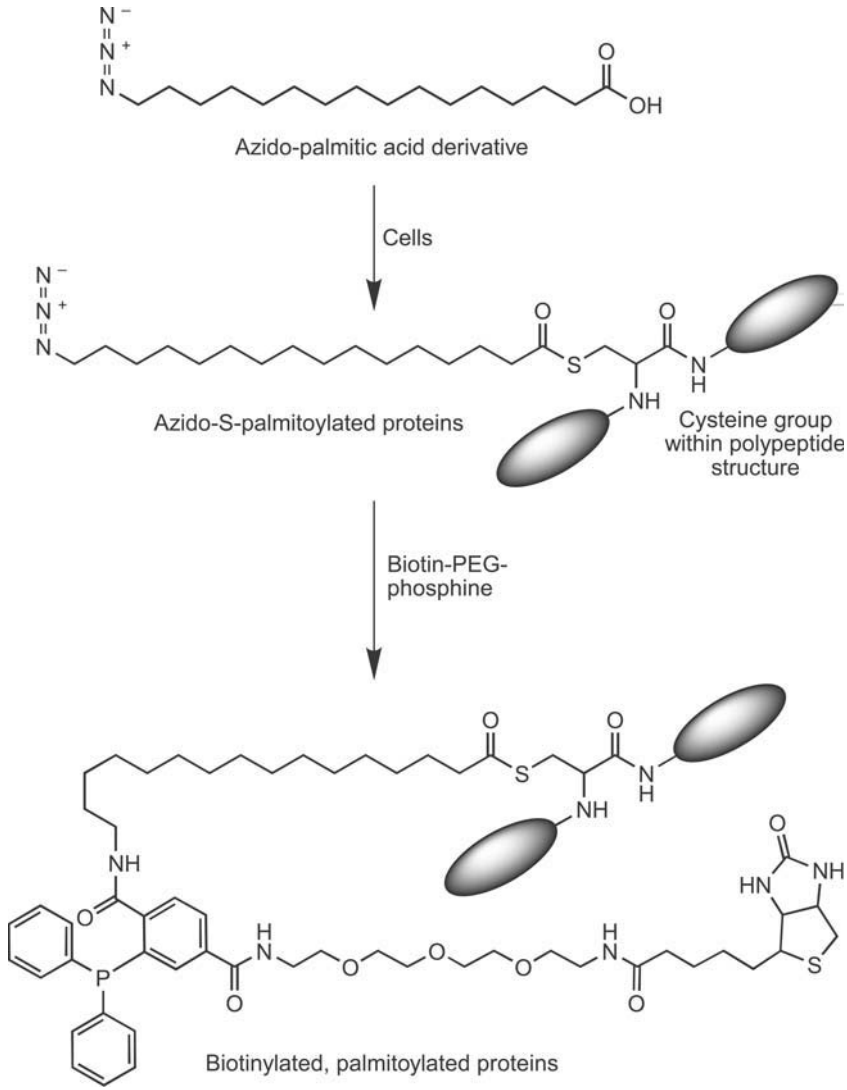


Figure 17.20 An azido-palmitic acid derivative can be added to cells to obtain palmitoylated proteins that contain an azide group able to participate in the Staudinger ligation reaction. Biotinylation of these post-translationally modified sites then can be done *in vivo* using a biotin-phosphine reagent.

peptide. This enzyme uses a farnesyl diphosphate derivative to transfer covalently the lipid to the cysteine residue via a thioether bond. Xu *et al*. (2006) found that the use of 6,7-dihydrogeranylazide diphosphate resulted in appending the terminal azido derivative onto such peptides, yielding an azido functionality for subsequent conjugation using Staudinger ligation. This process enabled targeted coupling through the C-terminal of any peptide or protein containing the CAAX box sequence.

Another important variation of the Staudinger ligation reaction described above involves the use of cleavable aryl groups on the triphenylphosphine component, which allows for

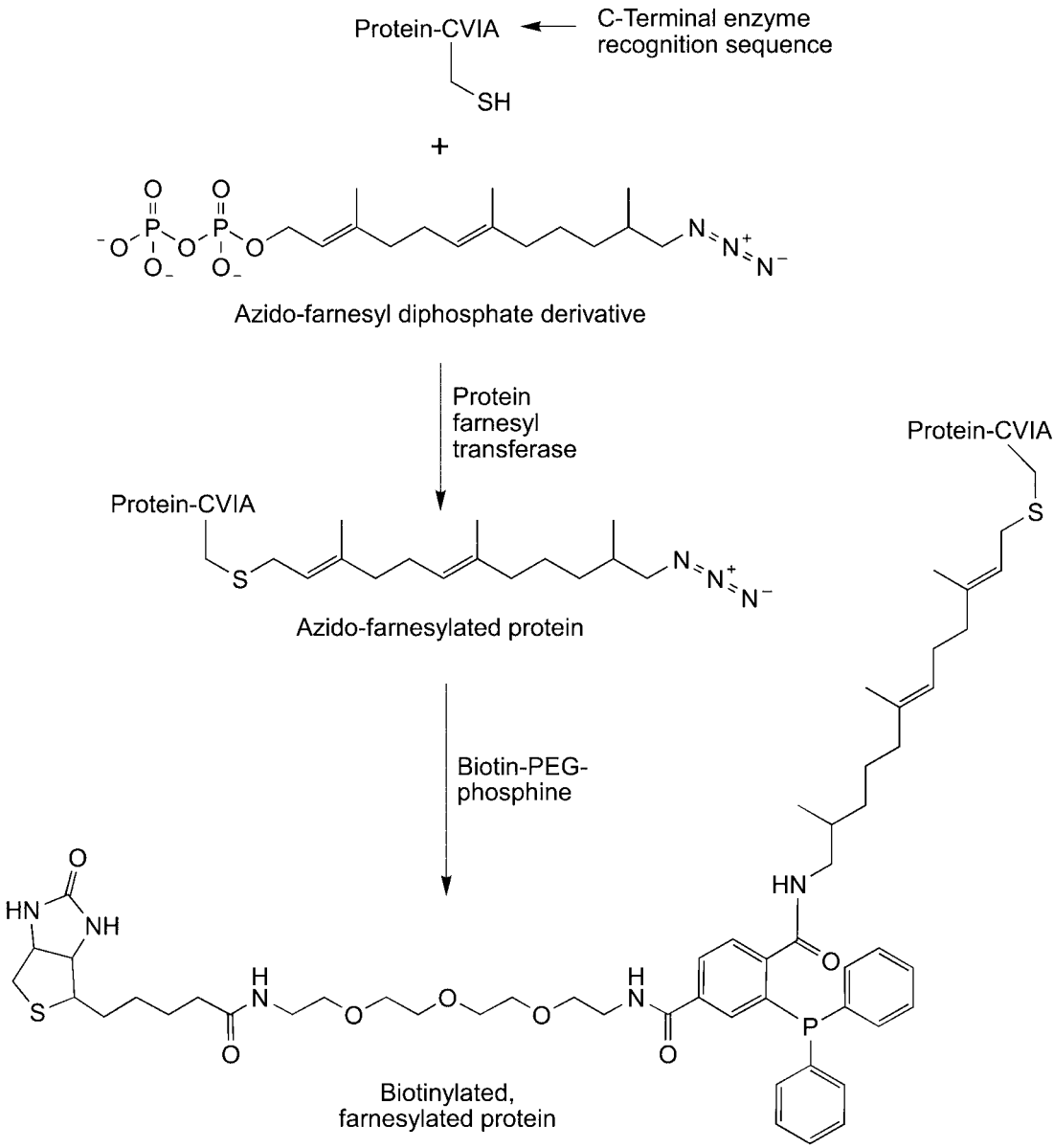


Figure 17.21 An azido-farnesyl diphosphate derivative can be added to cells to obtain farnesylated proteins containing terminal azide groups that can be targeted in a Staudinger ligation reaction. Biotinylation of these post-translationally modified proteins can be done *in vivo* using a biotin-phosphine derivative.

a "traceless" ligation reaction to occur. This strategy results in a zero-length amide bond between the phosphine derivative and the azide derivative, thus removing the triphenylphosphine component from the final conjugate and linking the two molecules together directly (Nilsson *et al.*, 2000, 2001; Saxon *et al.*, 2000; Soellner *et al.*, 2002; Saxon and Bertozzi, 2003, 2006).

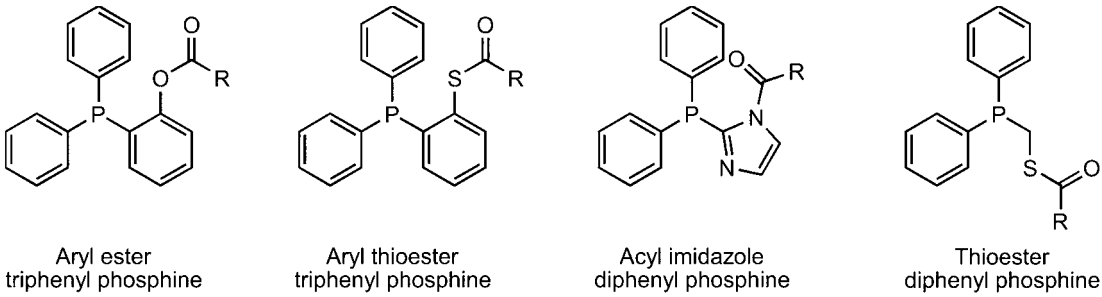


Figure 17.22 Certain unique phosphine derivatives can be used in the design of modification or conjugation reagents to create a traceless Staudinger ligation process, wherein the phosphine group is lost and an amide bond between an azide-containing molecule and the phosphine-containing molecule results.

The phosphanes useful in this process are built from acyl derivatives of compounds such as those shown in Figure 17.22. During the Staudinger ligation process, once the azide reactant forms the aza-ylide with the phosphine, electrophilic attraction induces the nitrogen to attack the electron deficient carbonyl, which in turn causes release of the phosphonium group and forms the amide bond (Figure 17.23).

The most useful phosphane derivatives in a traceless Staudinger reaction include the acyl modified 2-diphenylphosphanylphenol and the acyl modified diphenylphosphanylmethanethiol. These two core structures provide the best rate of reaction and efficiently form the amide bond conjugate (Köhn and Breinbauer, 2004). The traceless Staudinger ligation method no doubt will become a popular choice to avoid retention of the bulky phosphane species in bioconjugates. The reaction can be used with success to produce long polypeptide chains by linking together azido-peptides with phosphine-peptides to form the appropriate biological peptide bond between them (Nilsson *et al.*, 2003). This makes it possible to create synthetically polypeptides that are too long to create using standard solid phase peptide synthesis procedures (Figure 17.24). In addition, the traceless Staudinger ligation reaction can be used to link biomolecules containing azide groups to surfaces containing the traceless phosphane derivative (Soellner *et al.*, 2003).

6. Native Chemical Ligation

Native chemical ligation is an important alternative chemoselective peptide conjugation technique to the previously discussed non-native methods (Dawson *et al.*, 1994). This system can provide discrete coupling of the N-terminal of one peptide to the C-terminal of another peptide using a unique reaction process, essentially extending a peptide chain while maintaining native sequence and bonding characteristics. The reactant partners are prepared through typical peptide synthetic procedures, wherein one peptide is made to contain an α-thioester on its C-terminal carboxylate and the other peptide is synthesized to contain a cysteine amino acid residue at its N-terminal. The reaction of these two derivatives proceeds through nucleophilic attack of the cysteine thiol of one peptide onto the carbonyl group of the α-thioester at the C-terminal of a second peptide. The result forms an intermediate thioester by transthioesterification, which then spontaneously rearranges by an S → N acyl transfer to form a native amide bond between the peptides with no foreign organic structure remaining (Figure 17.25). The reaction proceeds

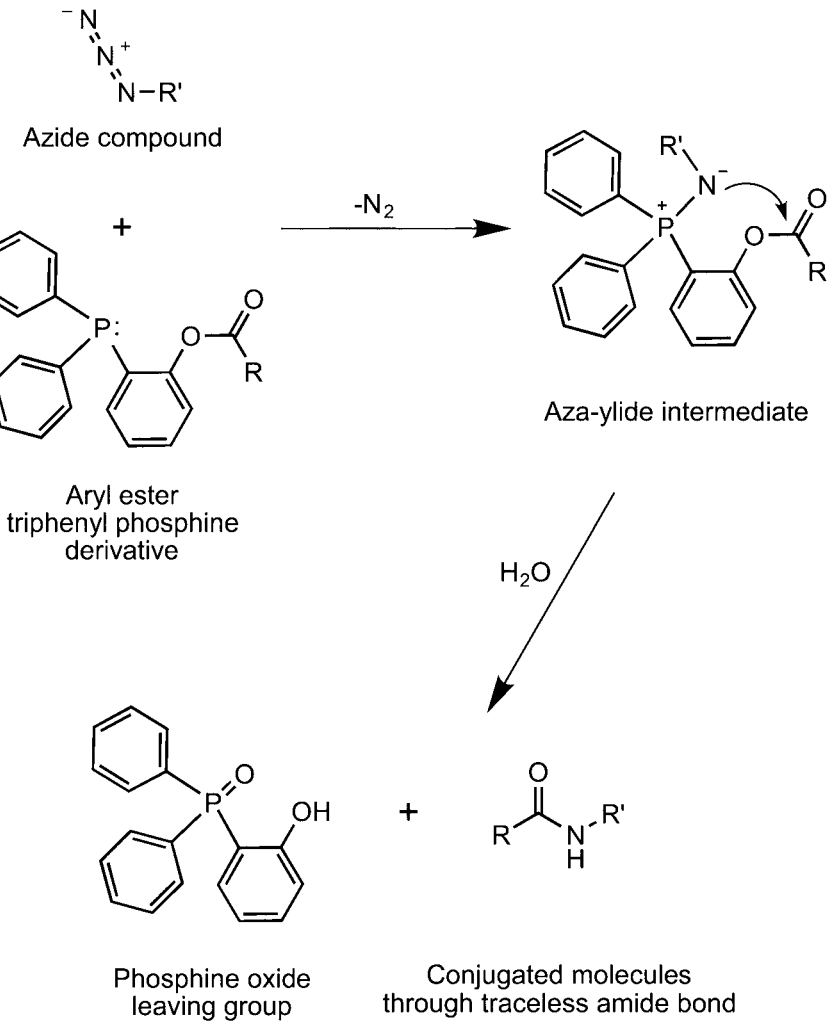


Figure 17.23 A traceless Staudinger ligation process involves the formation of an intermediate aza-ylide with subsequent attack of the nucleophilic nitrogen atom on the neighboring electrophilic group. The formation of an amide bond then occurs concomitant with the loss of the phosphine component, thus forming a zero-length crosslink between the two molecules.

at physiological pH using fully unprotected (i.e., unblocked amino acid functional groups), which is unusual in peptide synthetic strategies.

Peptides typically are prepared for this ligation process using α-alkyl thioesters, because they are simple to make at the time of peptide synthesis. However, due to the relatively slow reaction kinetics of alkyl thioesters, most native chemical ligation processes have been catalyzed through the use of thiol compound additives, such as benzyl mercaptan or thiophenol (Dawson *et al.*, 1997). These compounds react with the initial α-alkyl thioester to form another intermediate, an aryl thioester, which is more reactive toward the N-terminal cysteine on the other peptide to be coupled. A study

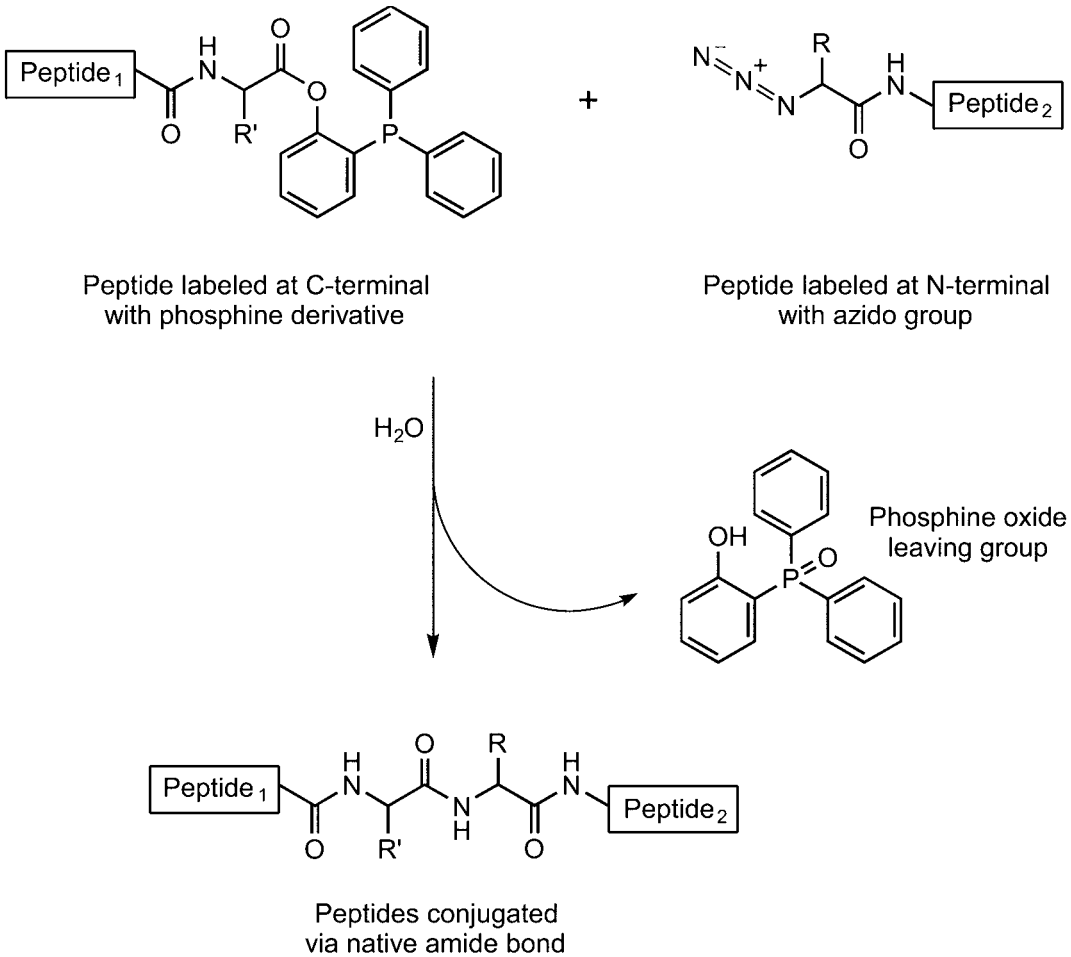


Figure 17.24 The traceless Staudinger reaction can be used to form larger peptides from smaller peptides, if one contains an azido group at the N-terminal and the other one contains a phosphine ester at its C-terminal. The reaction gives a native peptide (amide) bond with loss of the phosphine group.

of the rate of reaction for different thiol catalysts indicated that (4-carboxymethyl)thiophenol performed the best. The addition of this compound to a native chemical ligation reaction resulted in at least a 10-fold improvement in reaction rates (Johnson and Kent, 2006).

Another advantage to the use of a thiol additive is that the abundance of free thiol groups in the reaction environment will prevent the oxidation of the cysteine thiol at the N-terminal of the other peptide. Without added thiol transesterification catalysts, disulfide formation resulting in dimerization of the Cys-peptide would be a dominant side reaction in aqueous, oxygenated buffer conditions.

Native chemical ligation has been used successfully to couple two unprotected peptides together during solid phase synthesis, wherein one of the peptides is attached to the resin using a thioester linkage and the other peptide is introduced containing a cysteine at its N-terminal

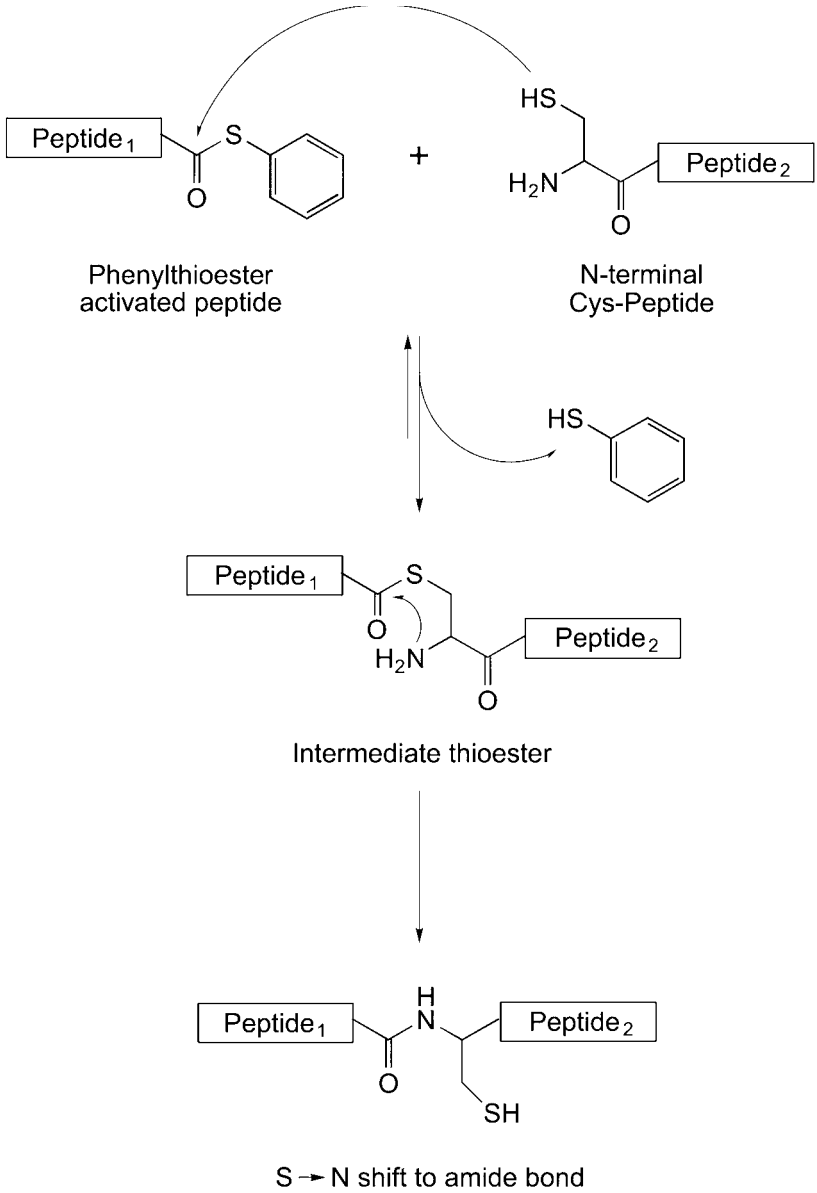


Figure 17.25 The native chemical ligation reaction can be used to form larger peptides from smaller peptides, if one contains a cysteine residue at its N-terminal and the other one contains a thioester on its C-terminal. Reaction of the peptide derivatives gives a native peptide (amide) bond.

(Camarero *et al.*, 1998). The ligation process also could form cyclic peptides, although the efficiency of the reaction was less than the conjugation of two separate peptides.

So efficient is this method to link peptide sequences together in a native amide bonded state that complete proteins of various sizes have been synthetically made (Hackeng *et al.*, 1999),

such as the preparation of a serine protease (Pal *et al.*, 2003), bovine pancreatic trypsin inhibitor (Lu *et al.*, 1998), cytochrome b562 (Low *et al.*, 2001), and the triple zinc finger protein, Zif268 (Beligere and Dawson, 1999). Many other examples of full or partial protein synthesis can be found in the published literature (for reviews, see Dawson and Kent, 2000; David *et al.*, 2004).

Native chemical ligation also can be extended to the conjugation of peptides or proteins to other molecules or surfaces. For instance, Reulen *et al.* (2007) prepared liposomes that contained cysteine–PEG–phospholipid derivatives and then coupled thioester-modified peptides or proteins to form a protein–liposome conjugate. Using this procedure, approximately 100 molecules of a collagen binding protein could be coupled to the cysteine-containing liposomes.

In addition, Dose and Seitz (2005) employed native chemical ligation to synthesize peptide nucleic acids (PNAs) by linking shorter segments of PNAs to make long contiguous strands, which could not be made through typical oligo synthesis procedures.

6.1. Expressed Protein Ligation and Inteins

Although there are a limited number of reports indicating that N-terminal cysteine-containing proteins can be expressed recombinantly, the *in vivo* production of a protein having a C-terminal thioester is considerably more difficult. That's why the initial development of native chemical ligation techniques was restricted to the use of peptide synthesis to generate the two peptide derivatives for ligation. However, the recombinant generation of C-terminal thioesters and N-terminal cysteine residues in peptides for native chemical ligation now can be done using intein technology. In the same frame that native chemical ligation reactions were being discovered and explored, intein technology was being investigated as a new *in vivo* native protein splicing and ligation mechanism.

Inteins are certain sequences of amino acids in precursor proteins that can catalyze a cleavage of their own internal peptide structure, resulting in the excision of the intein peptide segment and ligation together of the two peptide segments flanking the excised region (exteins). The exteins are thus ligated together with a native peptide (amide) bond. The immediate C-terminal side flanking the intein usually contains either a serine/threonine residue or a cysteine amino acid group at the splice junction. The N-terminal extein side next to the intein also can contain either a serine/threonine group or a cysteine residue, which are able to form an ester or thioester functionality, respectively. If a cysteine group is present at the N-terminal splice junction side of the intein, then the thioester intermediate that forms is very similar to the reaction intermediate of native chemical ligation.

The intein segment is excised through a number of steps that first involves an N → S shift at the C-terminal cysteine residue at the extein junction to create a thioester intermediate. Then nucleophilic attack of either the serine/threonine hydroxyl or the cysteine thiol of the extein on the N-terminal side of the intein proceeds to cleave the C-terminal extein and ligate it to the N-terminal extein thiol (or hydroxyl) through another (thio)ester bond. Therefore, the cleavage and ligation reaction proceeds through a second ester or thioester intermediate that involves either an O→N shift or an S→N shift, which cleaves off the intein segment entirely. The complete thioester reaction route is shown in Figure 17.26, which can be seen to be very similar to that of a native chemical ligation reaction, illustrated previously.

Muir *et al.* (1998) realized that the intein reaction could be used to facilitate a native chemical ligation with a synthetic N-terminal cysteine-containing peptide or cysteine-containing molecule. With the discovery of a mutant intein that could form an intermediate thioester but not go on to complete the splice and ligation reaction (Xu and Perler, 1996; Chong *et al.*,

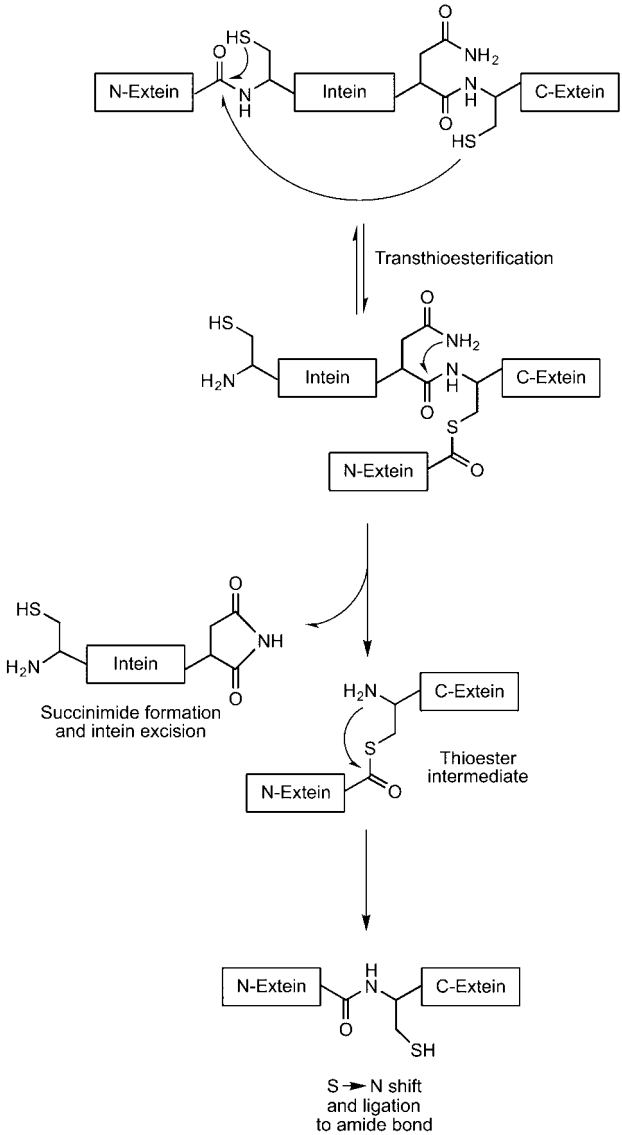


Figure 17.26 The native process leading to intein excision and ligation of extein fragments involves a sequence of reactions involving transthioesterification, cleavage of the intein fragment, and a S → N shift, which ligates the two extein peptides together via an amide bond.

1997), this expression technology now could be combined with the native chemical ligation reaction to facilitate a new Expressed Protein Ligation (EPL) method.

Fusion vectors are available that combine a recombinant protein with a mutant mini intein segment (not containing an endonuclease domain) and followed by a chitin binding domain (CBD; Zhang *et al.*, 2001). These mutants typically also have an alanine substitution that replaces the cysteine or serine/threonine usually found on the C-extein splice junction. Alanine

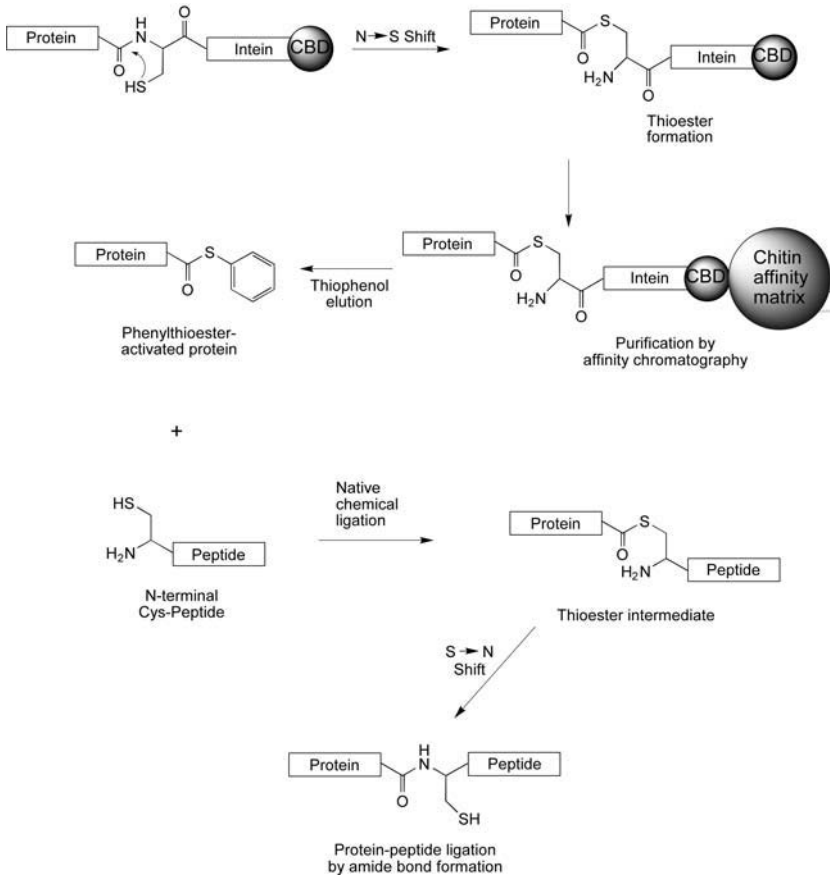


Figure 17.27 The EPL process involves a fusion protein containing an intein tag plus a CBD. The fusion protein is captured on an immobilized chitin resin and after removal of contaminating proteins, it is eluted using thiophenol, which cleaves at the thioester bond between the intein and the desired expressed protein. This releases a phenylthioester-activated protein that can be used in the native chemical ligation reaction with another peptide containing an N-terminal cysteine residue. Conjugation results in a native amide (peptide) bond formed between them.

cannot facilitate attack on the thioester on the N-extein side and thus the intein is not cleaved. Using the CBD portion, the entire expressed fusion protein can be purified on a chitin column through specific affinity binding and then the N-extein segment induced to cleave using DTT, which results in release of the desired recombinant protein having a C-terminal thioester.

However, if the expressed protein is treated on the affinity support using thiophenol, this also will release the protein and result in a phenylthioester at its C-terminal, which is the reactive intermediate imminently suitable for native chemical ligation. Treatment of this activated thioester protein with a N-terminal cysteine peptide induces the native chemical ligation reaction and couples the peptide to the expressed protein through an amide bond (Severinov and Muir, 1998) (Figure 17.27).

EPL extends the applicability of native chemical ligation to recombinantly produced proteins using the mutant mini intein vector system. Proteins being expressed using this method will

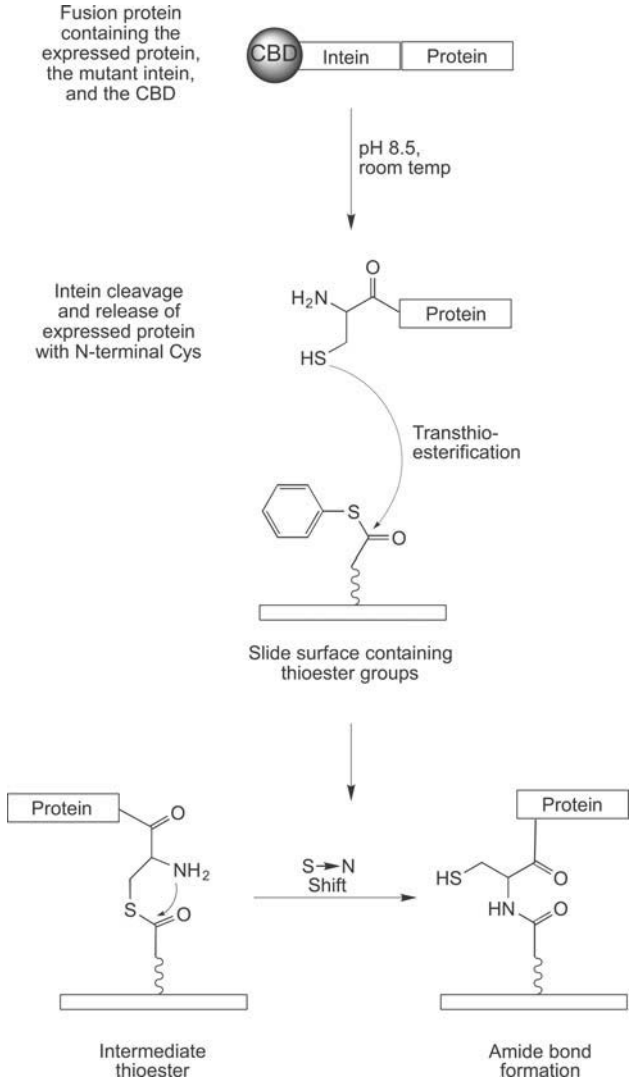


Figure 17.28 EPL reactions can be used to couple a fusion protein to a surface containing a thioester derivative. After cells are grown and the fusion protein expressed, a pH and temperature shift causes intein cleavage with release of the expressed protein with an N-terminal cysteine residue. Reaction with the thioester surface results in a native chemical ligation reaction that forms an amide bond linkage with the expressed protein.

contain a C-terminal thioester and therefore can be conjugated to any reagent or probe molecule containing a cysteine group with an available α-amine and thiol group. Expressed proteins also can be immobilized onto solid surfaces by coupling them solely at their C-terminal ends.

Girish *et al.* (2005) coupled proteins onto surfaces using this approach, but in this case expressing a protein containing an N-terminal Cys residue and then reacting it with a thioester group on a glass slide (Figure 17.28). A similar strategy also has been used with success to label specifically expressed proteins in live cells using thioester-containing probes (Nilsson *et al.*,

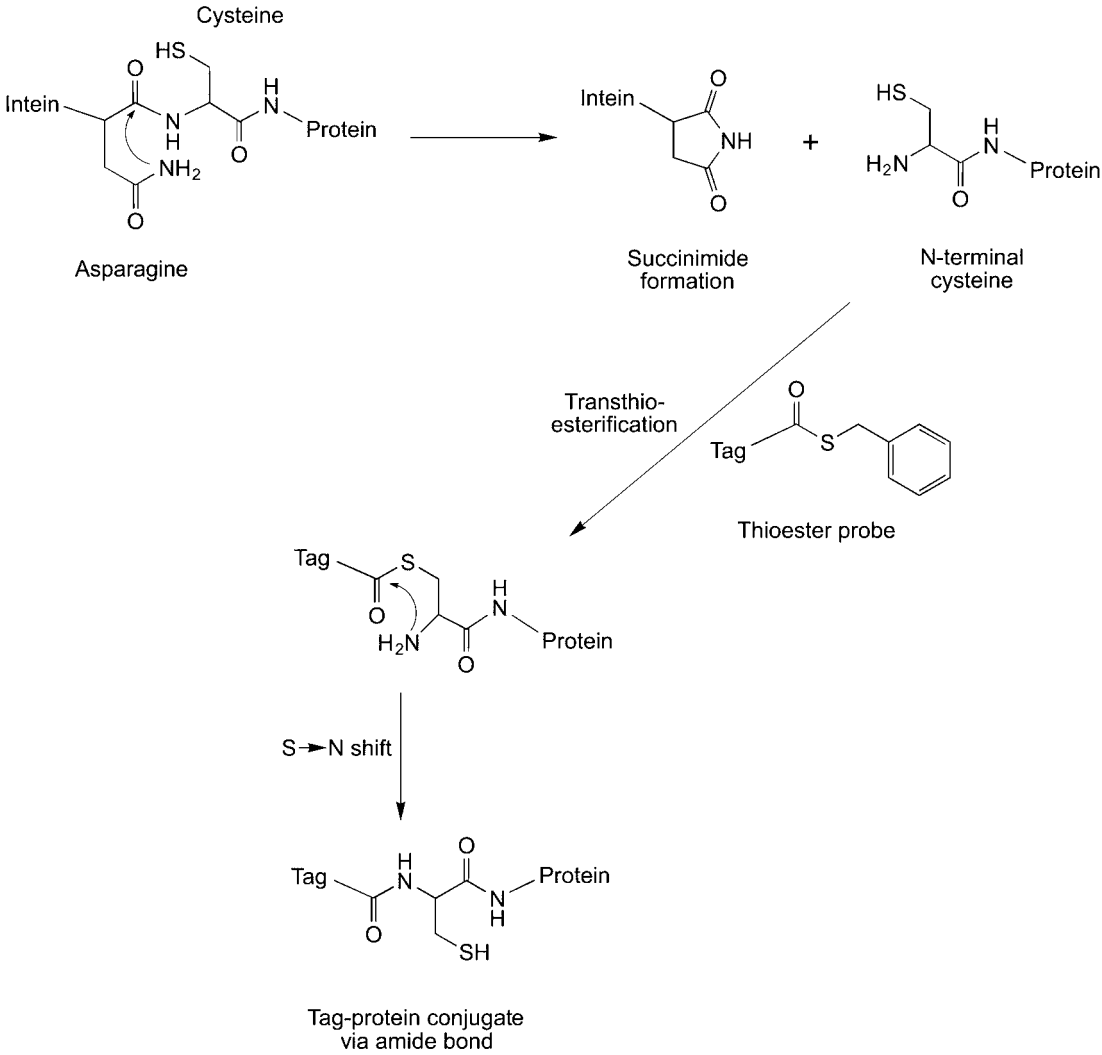


Figure 17.29 An expressed protein containing a mutant intein segment can undergo self cleavage to form an N-terminal cysteine residue, which then can be reacted with a thioester probe to label specifically the protein via an amide bond.

2003). Using the pTWIN vector (New England Biolabs), a recombinant system was developed to express proteins having an N-terminal Cys using the Ssp DnaB mini intein segment (Yeo *et al.*, 2003). This intein contains a C-terminal asparagine residue that undergoes a self-cleaving reaction after a shift in pH and temperature. Growing cells in physiological pH conditions does not cleave the intein. However, after cell lysis, an increase to pH 8.5 under room temperature conditions will initiate the cleavage reaction. Thioester-containing probes, including thioester-fluorescent molecules or thioester–biotin, can be added directly to live cells to label these expressed proteins via native chemical ligation (Figure 17.29).

The following protocol for EPL, including purification using a CBD fusion tag followed by native chemical ligation, is based on the methods of Muir *et al.* (1998), Chong *et al.* (1997, 1998), Evans *et al.* (1998), Severinov and Muir (1998), and the NEB instruction manual for the IMPACT-TWIN system. The recombinant protein is recovered from the affinity column as the thioester derivative ready for reaction with a N-terminal Cys peptide or another tag containing a Cys residue.

Protocol

1. A gene encoding a recombinant protein of interest is cloned into a cleavable mutant intein-CBD plasmid vector and transfected into cells (e.g., using the IMPACT-TWIN system from New England Biolabs). The cells are grown in LB media with 200 μg/ml ampicillin at 37°C to an $OD_{600\,nm}$ of about 0.5–0.8, induced to express the fusion protein complex by the addition of 1 mM IPTG, and incubated overnight.
2. Prepare a chitin affinity column by washing with at least 10 bed volumes of 25 mM HEPES, 250 mM NaCl, 1 mM EDTA, 0.1 percent Triton X-100, pH 7.0 (wash buffer).
3. Recover the cells by centrifugation, lyse them in wash buffer containing protease inhibitors (e.g., 20 μM PMSF), and clarify the supernatant by centrifugation.
4. Apply the lysate onto the affinity column and wash with at least 10 column volumes of wash buffer to remove not-bound protein. Monitor the eluate by absorbance at 280 nm to assure that baseline has been reached.
5. Wash the column with 2–3 column volumes of elution buffer (25 mM HEPES or Tris-HCl containing 500 mM NaCl, 1 mM EDTA, and 2 percent (v/v) thiophenol (or 50 mM 2-mercaptoethansulfonic acid), pH 8.5. Next, add 2 column volumes of elution buffer containing 1–2 mM of an N-terminal Cys peptide or Cys-containing tag. Stop the flow and incubate the column at room temperature for 24 hours. The thiophenol will cleave the recombinant protein at the intein splice junction, while the cysteine-containing peptide will react with the intermediate ester through transthioesterification and an S → N shift to ligate the molecules via an amide bond.
6. Elute the conjugated molecules using wash buffer and purify using dialysis or gel filtration.

18

Discrete PEG Reagents

Poly(ethylene glycol) (PEG) has been used for many years as a modification and conjugation reagent for biological molecules (Roberts *et al*., 2002). Part of the rationale for using PEG polymers in bioconjugation applications includes a dramatic increase in the water solubility of modified molecules, a decrease in immunogenicity due to the shielding of modified molecules from the immune system, protection of modified protein and peptides from digestion by proteolytic enzymes, and effectively increasing their hydrodynamic volume and decreasing clearance rates by renal filtration, all of which results in an increase in the serum half-life of modified molecules *in vivo*. Thus, hydrophobic drugs modified with PEG polymers become much more water–soluble, foreign proteins and other immunogenic molecules are hidden from the circulating antibodies and cells of the immune system, labile peptides or proteins can't be degraded by enzymes, and the reticuloendothelial system and kidneys can't remove labeled drugs as quickly as their unlabeled counterparts. For these reasons, many drug candidates using PEG modification currently are in clinical trials and several are already on the market.

The majority of PEG applications involve the use of long-chain linear or branched PEG polymers having a molecular mass of at least 2.5 kDa to over 50 kDa (see Chapter 25 for selected applications of these long PEG polymers). PEG typically is made from ethylene oxide by an anionic ring-opening reaction, which results in long polymer molecules consisting of the general structure HO—$(CH_2CH_2O)_n$—H. The number of repeat units (n) in standard commercial PEG polymers created through this process can be anything from less than 50 to over 1,100. However, in these conventional PEG polymers of any given size, there actually exists a distribution of chain lengths, as is typical for any polymer-based substance. The range of chain lengths for a given PEG size is approximately Gaussian in distribution, which means that most PEG reagents prepared by standard polymerization processes are fairly disperse. The level of polydispersity is usually indicated by Mw/Mn, which is called the polydispersity index (PDI) and is equal to the weight average molecular weight (Mw) divided by the number average molecular weight (Mn). If the PDI is equal to 1, the polymer is said to be monodisperse. For PEG polymers, the PDI is typically less than 1.2, which is quite good for polymeric materials, and this value probably reflects the concern over the use of polydisperse PEG for therapeutic applications. However, even at this low level of PDI, the chain distribution often is quite high. Kenworthy *et al*. (1995) reported that the mean number of repeat units and variance for PEG 2000 averages 53 units with a variance of 11, while a PEG 5000 polymer has an average 130 units with a

variance of 20. Shorter chain PEG polymers have a tendency to have greater polydispersity than the large polymers. For example, the commercially available PEG 1500 can have between 19 and 48 repeat units in a typical preparation, which correspond to a molecular weight distribution of 800–2,100 Da (Davis and Crapps, 2006). Thus, most commercial sources of crude PEG polymers are highly disperse and they probably should be avoided entirely for critical bioconjugation work, unless they have been carefully purified to isolate a single chain length.

However, true monodisperse PEG reagents have become available now, which are made not by polymerizing small monomers, but by linking discrete PEG segments together to create pure polymers of known structure and purity. These discrete PEG molecules can be made in chain lengths from as little as 2 to over 24 repeating ethylene oxide units, and theoretically, virtually any chain length can be produced by building up from smaller precursors (Davis and Crapps, 2006).

Using a convergent synthesis process, a short PEG segment containing a hydroxyl protecting group on one side and a free hydroxyl on the other end is reacted with another PEG segment containing a reactive group on one end and a protecting group on the other end. The reactive group also must be a good leaving group, so that once it reacts with the hydroxyl on the other PEG unit, conjugation reaction occurs to form an ether bond. This results in the covalent linking of the PEG molecules together to form a longer PEG compound equal to the combined length of the reactants. Deprotection of the ends then can be done to add additional functionality, such as a reactive group, functional group, or to form a hydroxyl or methoxy end.

Prior to this synthetic method being developed, small PEG-containing compounds were limited to very short ethylene oxide segments, such as the commonly used reagents ethylene glycol and tetraethylene glycol. Now using new discrete PEG reagents, the advantages that PEG compounds have provided for use in the modification and crosslinking of biomolecules can be incorporated at known polymer lengths into any bioconjugation reagent to enhance its properties.

Discrete PEG reagents have been reported that incorporate reactive groups, fluorescent probes, metal chelates, drug molecules, affinity ligands, biotin, and a host of other constituents. For instance, Wei *et al.* (2006) developed a PEG-functionalized texaphyrin derivative, which was shown to have enhanced solubility and anti-cancer activity *in vivo*. Four $mPEG_4$ chains decorating the central gadolinium(III) texaphyrin were found to convey dramatic anti-proliferative effects compared to the parent chelate without PEGs present.

In another application of a PEG_4 spacer, Clevenger *et al.* (2004) prepared a biotinylated derivative of the antibiotic geldanamycin (GDA) to use as an inhibitor of the 90 kDa heat shock protein Hsp90. Use of the PEG linker in building such an organic drug complex has the advantage of adding a hydrophilic arm to an otherwise very hydrophobic probe. The biotin–PEG_4–GDA conjugate could be used to bind the active site of Hsp90 proteins and then affinity purify them on a (strept)avidin-containing resin.

Similarly, Kruszynski *et al.* (2005) used the reagent NHS–PEG_4–biotin to make biotinylated analogs of human MCP-1. This compound, described later in this section, provides a long-chain biotin handle that has better solubility properties than the corresponding aliphatic reagent NHS–LC–biotin, which has been used in many applications (Chapter 11, Section 1). Kornilova *et al.* (2005) used the same PEG reagent to biotinylate various γ-secretase peptide inhibitors to create probes of this multi-protein complex.

Hydrophilic short biotin–PEG tags also have found their way into the design of multifunctional crosslinkers to study protein structures by mass spec. Fujii *et al.* (2004) developed a homobifunctional NHS ester crosslinker that in addition has a PEG–biotin handle (Figure 18.1). The reagent actually is a trifunctional compound similar to the biotinylated PIR compound

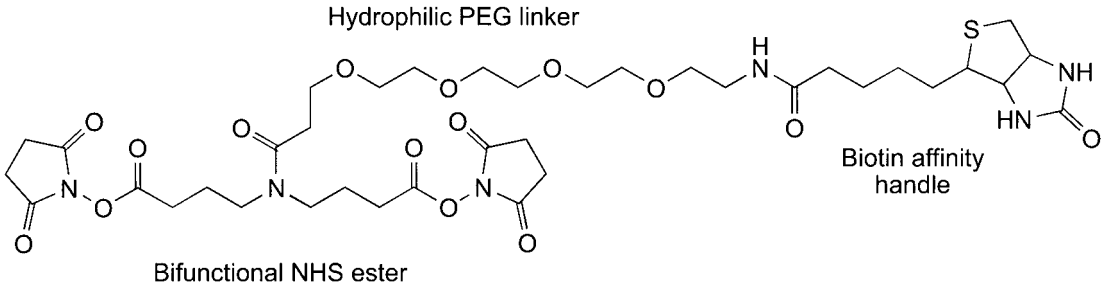


Figure 18.1 A trifunctional reagent for studying protein interactions by mass spec. The bis-NHS ester arms crosslink interacting proteins, while the discrete PEG-containing biotin arm can be used to isolate or detect the conjugates using (strept)avidin reagents.

described in Chapter 28, Section 1.4. The NHS ester arms are of identical length to provide linkages of the same molecular distance in each direction, while the PEG group on the biotin arm avoids the hydrophobic collapse of alkyl chain spacers by providing an extremely hydrophilic linker with high freedom of motion in aqueous environments. Due to the increased water solubility provided by the PEG linker arm, the sensitivity of mass spec analysis was enhanced over the use of more traditional aliphatic spacers. In particular, this discrete PEG compound was found to be optimal for performing mass spec analysis in three-dimensions (MS^3).

Coupling of affinity molecules to surfaces also can be enhanced by the use of discrete PEG linkers. Nishimura *et al.* (2005) modified an amino surface with a NHS–PEG_{12}–maleimide crosslinker to create a hydrophilic self-assembled monolayer (SAM) surface that was thiol reactive for the conjugation of sulfhydryl-modified RNAs. This array then was used to investigate the binding specificity of synthetic kanamycins with selected RNA sequences to prove the specific interaction of ribosomal RNA with this molecule. The PEG linkers on surfaces provide lower nonspecific binding character than alkyl linkers, when preparing SAM surfaces for affinity interactions.

The low nonspecificity of PEG layers also was used to eliminate biomolecule binding to certain areas of an array. Kidambi *et al.* (2004) patterned an mPEG-carboxylate molecule onto polyelectrolyte multilayers to mask portions of the surface. The extremely low binding character of PEG provides advantages for creating patterned surfaces that other modifiers using aliphatic alkyl linkers do not provide.

SAM surfaces on metals, such as gold particles or planar arrays, have been enhanced in their performance and properties through the use of PEG-containing modifications. Prime and Whitesides (1991) used an mPEG–thiol combined with a slightly longer thiol–PEG-carboxylate compound to create monolayers on gold through dative bonding with the thiol groups. The thiol–PEG-carboxylate compound typically is used at about 10 percent of the concentration of the thiol–mPEG compound to form a lawn of low-binding mPEG molecules, which is interspersed with enough carboxylates to provide sites for covalent attachment of affinity ligands or antibodies. Spangler *et al.* (2004) improved upon this strategy by creating a dendritic structure branching off from a central phenyl ring core and containing two thiol arms and a single PEG arm containing either an mPEG group or a PEG-carboxylate (Figure 18.2). The presence of two thiol groups increases the strength of the linkage with the gold surface, thus providing resistance to oxidative cleavage of the SAM surface.

In another application involving a study of biotin compounds for potential use *in vivo*, it was found that the preferred structures included molecules that contained PEG spacers to

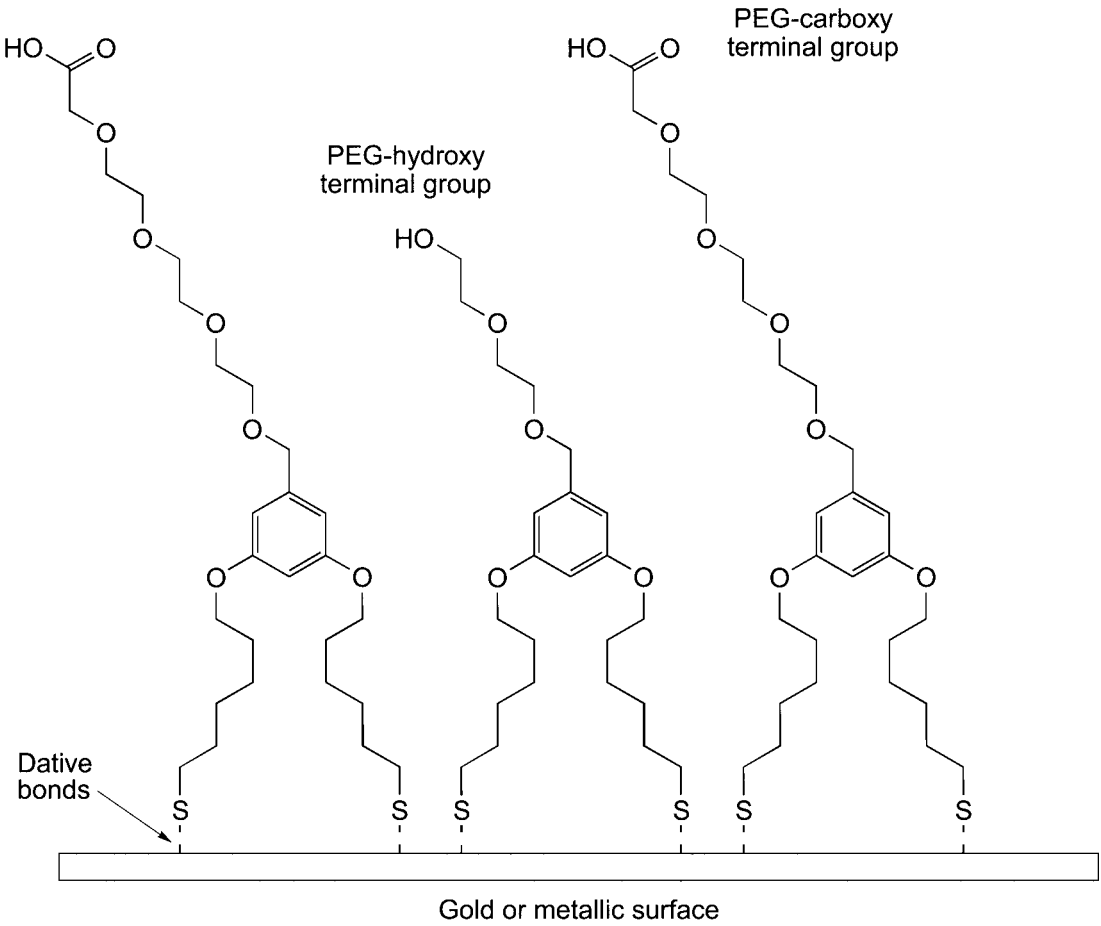


Figure 18.2 Dithiol linkers containing discrete PEG arms can be used to modify stably metallic surfaces for coupling biomolecules.

provide increased water solubility and *in vivo* activity (Wilbur *et al.*, 2000). Fifteen biotin derivatives were conjugated to cyanocobalamin, which is a binder of cobalt that may be used in radiotherapy for cancer. Structures with enhanced activity were formed with PEG spacers of varying length between the cyanocobalamin and the biotin handle, which provided maximal hydrophilicity to the entire complex.

Biotinylation reagents historically have used aliphatic chains to provide a spacer between a modified molecule and the bicyclic ring of biotin. This allows for enough molecular distance for (strept)avidin to easily bind during detection or targeting applications. Even long spacers on biotin reagents typically have been constructed of hydrophobic alkyl chains for the biotinylation of proteins. This can be detrimental to protein solubility, because biotin itself is sparingly soluble in aqueous solution and adding an alkyl spacer to this group only decreases its water solubility further. In particular, antibodies modified with long hydrophobic biotin compounds often aggregate and lose activity, especially at high modification levels. Replacing the

alkyl chain with a discrete PEG spacer, however, can dramatically increase water solubility and prevent antibody aggregation as well as significantly boost long-term stability.

Discrete PEG reagents can provide benefit for nearly any crosslinking compound or modification reagent designed for use in aqueous environments. Frequently, PEG chain lengths between 4 and 24 repeating ethylene oxide units create increasingly hydrophilic character for modified biomolecules or surfaces. The following sections describe a number of these PEG compounds, including crosslinkers, biotinylation compounds, and multi-armed PEG modification agents designed to block molecules and surfaces. In addition, PEG-based compounds are available that have functional groups on both ends for use as building blocks to create other PEG compounds or as spacers to build unique surface functionality. Most of these reagents are available commercially through Quanta BioDesign or Thermo Fisher Scientific.

Modification or crosslinking agents containing a discrete PEG spacer are often not powders or crystalline substances, but frequently they are thick, sticky, viscous liquids. Such materials are difficult to dispense by weighing out a small portion from a vial. For this reason, it may be best to dissolve an entire vial in a dry organic solvent prior to use or try to weigh out a much larger quantity than may be initially required. Stock solutions may be stored for weeks at $<0°C$, but long-term stability of active groups is dependent on the quality of the solvent used. For this reason, it is best to prepare solutions fresh.

Organic solvents that can be used with discrete PEG compounds include DMSO, DMF, DMAC (*N*,*N*′-dimethylacetamide), and methylene chloride. The compounds also are very soluble in many other commonly used organic solvents, which provide flexibility for doing reactions. DMAC is particularly convenient, because it is easily dried of contaminating water (using molecular sieves), it doesn't decompose like DMF (producing amines), and it doesn't have the odors of some of the other solvents. Methylene chloride can be used for water-insoluble molecules that are to be reacted with the PEG compounds, but don't require subsequent water miscibility.

1. Homobifunctional PEG Crosslinkers

Compounds having the same functionality on both ends are homobifunctional in nature and can be conjugated with the same target functionality on biomolecules, surfaces, or other molecules. Chapter 4 describes traditional homobifunctional compounds in detail, but the discrete PEG-based reagents are described here, because of their unique hydrophilic properties.

1.1. Bis-NHS Ester PEG Compounds

Bifunctional NHS esters can be used to conjugate two amine-containing molecules together. The NHS ester groups react with amines to form amide linkages with loss of NHS. Two different PEG spacer lengths are available in this type of reagent, a PEG_5 compound and a PEG_9 derivative (Figure 18.3). Unlike the popular BS^3 reagent (Chapter 4, Section 1.2), which is initially hydrophilic and water-soluble due to the presence of negatively charged sulfo-NHS esters on both ends but after reacting leaves behind a 6-carbon hydrophobic spacer, these PEG compounds attach two molecules together using a long and very hydrophilic bridge.

The bis-NHS–PEG_5 reagent provides a 21.7 Å spacer after attaching both ends to amines on two molecules, which is nearly twice the spacer distance provided by BS^3. The longer chain

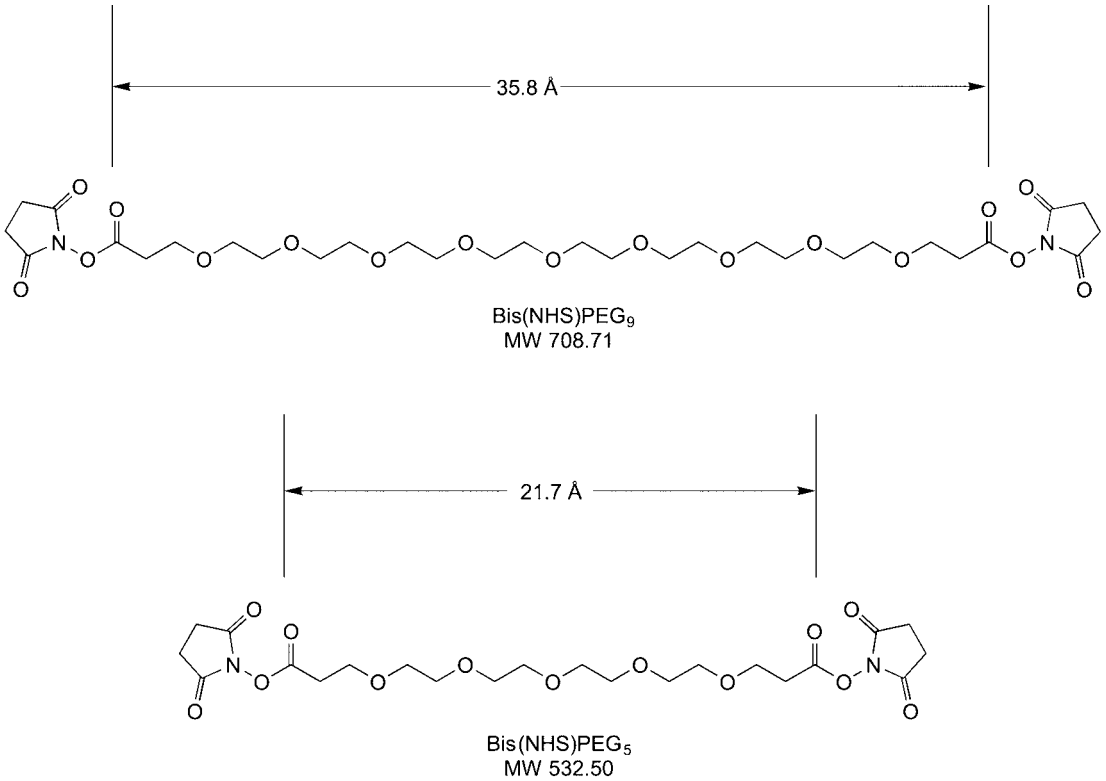


Figure 18.3 Homobifunctional NHS ester compounds containing PEG spacers for water solubility.

bis-NHS–PEG_9 reagent has a very long 35.8 Å spacer. All of these distances are measured between the carbonyl groups of the NHS esters, which would form the amide bonds and remain behind after crosslinking two molecules together. The distances represent the energy minimized, maximal molecular distance of a linear structure and may not reflect how such structures exist in solution.

The reaction of the NHS esters occurs at physiological pH or under slightly basic conditions to couple rapidly with amines and form amide bonds (Figure 18.4). The NHS ester groups also are subject to hydrolysis in aqueous solution, and the rate of hydrolysis increases with increasing pH. The more hydrophilic the molecule the greater is the potential for hydrolysis. Nectar reported that for long-chain polymeric PEGs containing NHS esters that the half-life of hydrolysis effectively triples upon lowing the pH one unit. In addition, the molecular constituents immediately adjacent to the NHS ester affect the half-life of hydrolysis of activated PEG compounds. For instance, the addition of a 4-carbon aliphatic chain between the PEG polymer and the NHS ester results in a half-life of hydrolysis of about 44 minutes at pH 8. Reducing this to only a 2-carbon unit decreases the half-life of the NHS ester to about 3 minutes. Therefore, to aid in NHS ester stability maintaining a reaction pH in the range of 7.0–7.5 is optimal for most applications.

The following protocol describes a general method for using bis-NHS ester PEG compounds. Optimization of concentrations should be done for each application to assure the best possible results. See also the protocol in Chapter 28, Section 1, which describes the use of homobifunctional

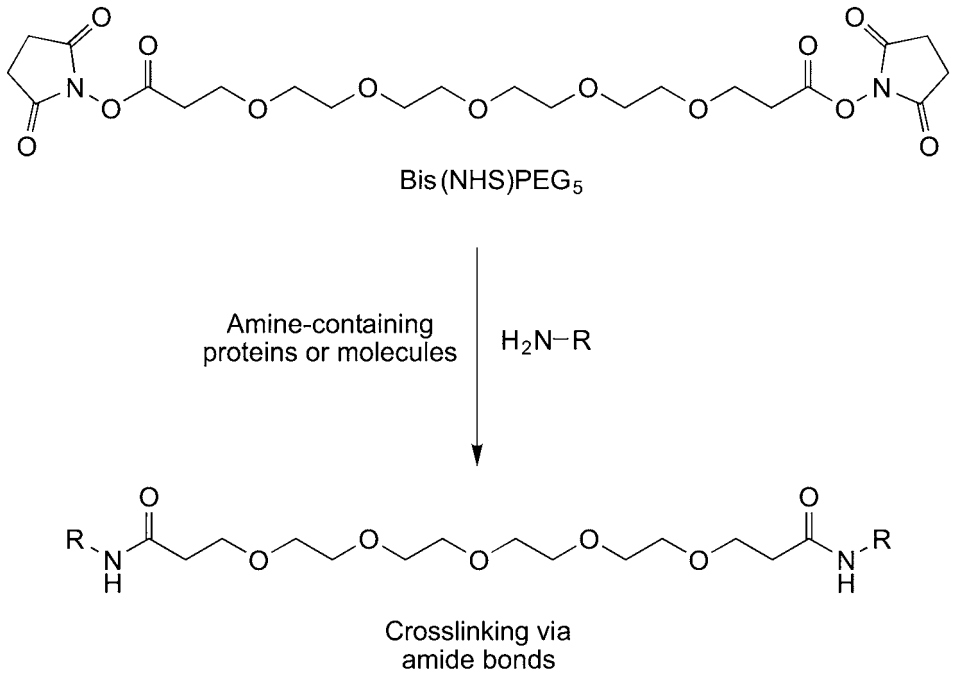


Figure 18.4 The reaction of bis-NHS–PEG_5 with amines on proteins yields amide bond linkages with amine-containing molecules.

NHS-ester compounds to study protein interactions. These bis-NHS–PEG compounds may provide a superior crosslinker for studying such interactions due to their water solubility and the fact that the PEG bridge won't get buried in hydrophobic pockets on proteins or within hydrophobic membrane structures.

Protocol

1. Dissolve the bis-NHS–PEG compound in a dry, water-miscible organic solvent to make a concentrated stock solution. To prepare a 10 mM solution of the bis-NHS–PEG_9 reagent, dissolve 7 mg/ml of DMAC; for the bis-NHS–PEG_5 reagent dissolve 5.3 mg/ml of DMAC.
2. Dissolve the molecules to be conjugated in 0.1 M sodium phosphate, pH 7.2 (for aqueous reactions) or in DMSO, DMAC, or methylene chloride (for organic reactions). If proteins are to be conjugated, a concentration of 1–10 mg/ml in buffer will work well in this protocol. For more dilute protein solutions, greater quantities of the bis-NHS–PEG compound may have to be added than recommended here to obtain similar levels of crosslinking.
3. Add a quantity of the crosslinker solution to the protein solution to provide a 1- to 10-fold molar excess of reagent over the concentration of protein. The use of lower molar ratios will limit the potential for oligomerization of proteins in solution. A series of reactions using different concentrations of crosslinker may have to be done to determine the optimal level to use for a particular application.
4. React for 30–60 minutes at room temperature.
5. Remove excess crosslinker by dialysis or gel filtration.

The bis-NHS–PEG compounds also may be used for modifying surfaces or particles that contain amine groups. If the bis-NHS–PEG reagent is reacted in large excess to the concentration of amines, then a single end of each crosslinker will couple to the amine groups on the surface and result in a PEG spacer terminating in a reactive NHS ester for further conjugation. Surfaces modified with an amino silane group, such as 3-aminopropyltriethoxysilane (APTS) are particularly good for further modification with PEG-based reagents. For instance, APTS modified glass slides or silica particles contain aminopropyl groups that can be modified with the bis-NHS–PEG compounds. This will create a hydrophilic monolayer having low nonspecific binding character and that can be used to covalently link to biomolecules via the terminal NHS ester group (Figure 18.5).

The following procedure can be used with planar or spherical surfaces containing amine groups. The reaction is done in organic solvent to preserve the activity of the terminal NHS ester after modification of the surface. For particle modification, care should be taken in choosing a solvent that won't damage the particle core structure, such as the potential for certain organic solvents to dissolve polymeric particles. Silica particles, however, are very robust to solvent conditions and can be used without damage. See also Chapter 14 for other particle conjugation methods.

Protocol

1. In ad fume hood, wash the amine-surface with organic solvent to remove any contaminants or water (especially when working with particles). Suggested solvents to use for this reaction are highly pure and dry DMAC, DMSO, or DMF. Particles can be washed by repeated centrifugation and resuspension.
2. Dissolve bis-NHS–PEG_5 into the solvent of choice at a concentration of 1 mg/ml also containing an equal molar concentration of triethylamine as base. Add the crosslinker solution to the surface or to the particles to coat them fully. When working with particles, centrifuge them to remove solvent prior to resuspending in the crosslinker solution.
3. React for 30–60 minutes at room temperature with mixing.
4. Wash the surface or particles with solvent at least thrice to remove excess crosslinker.

Planar surfaces activated with the NHS–PEG groups may be sealed in a pouch and stored dry in the presence of a desiccant. Activated particles may be stored as a suspension in dry solvent under a head of nitrogen at 4°C until used for further conjugation. The addition of an amine-containing protein or other amine-molecule will cause covalent coupling to the surface NHS ester groups to form amide bonds.

1.2. Bis-Maleimide–PEG Compounds

Homobifunctional crosslinkers containing thiol-reactive maleimides on each end of a PEG spacer are available in several sizes. These compounds are hydrophilic and react with sulfhydryls to produce thioether linkages, which are stable under most conditions. The following compounds can be obtained from Thermo Fisher or Quanta BioDesign.

$BM(PEG)_2$, $BM(PEG)_3$, and Bis-MAL–$dPEG_3$

$BM(PEG)_2$ is 1,8-bis-maleimidodithyleneglycol, a hydrophilic reagent containing a 14.7 Å spacer arm. This compound also is called $BM(PEO)_2$ by Thermo Fisher, wherein PEO is the acronym

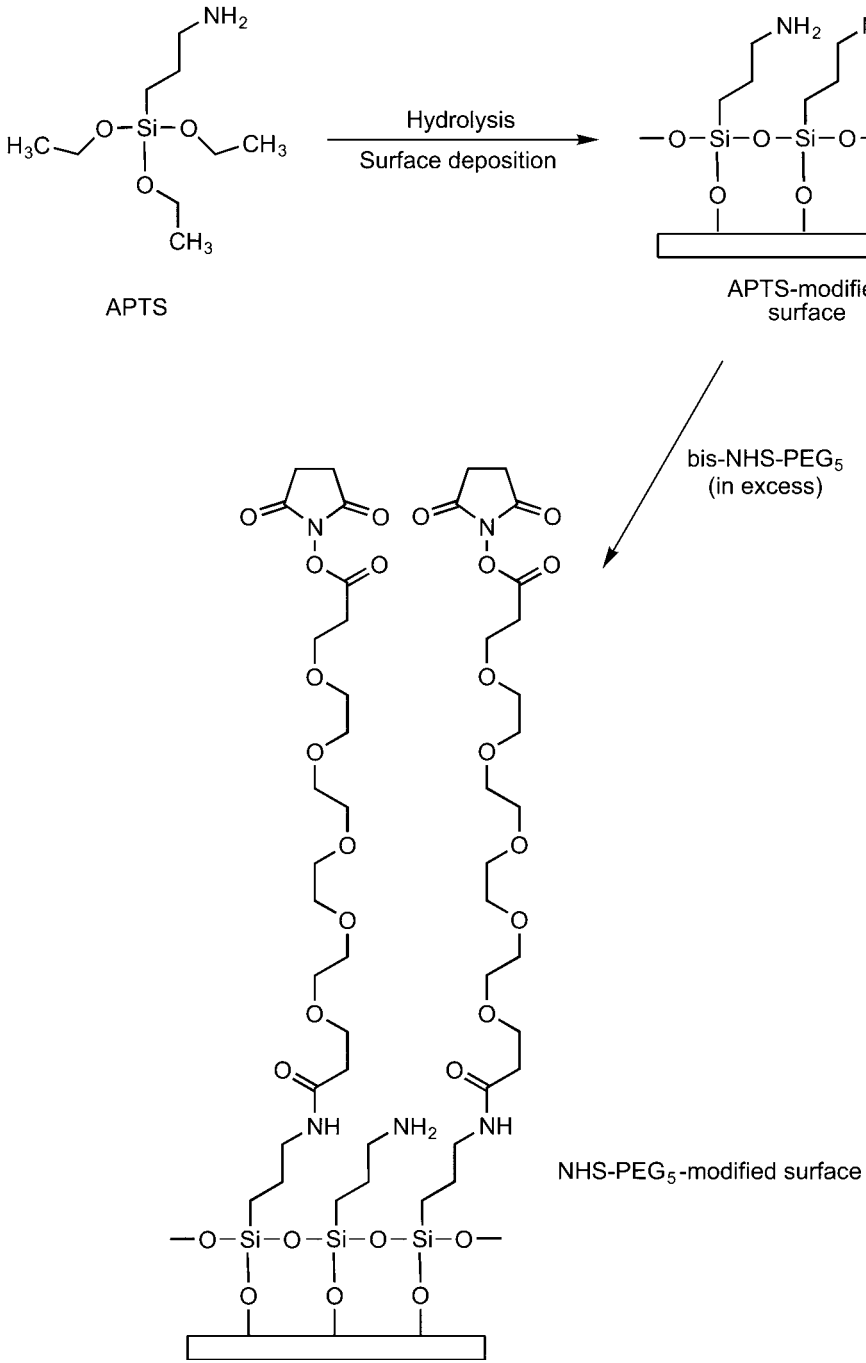
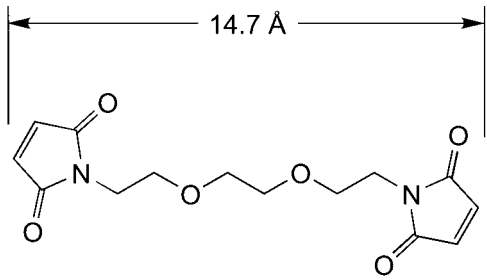
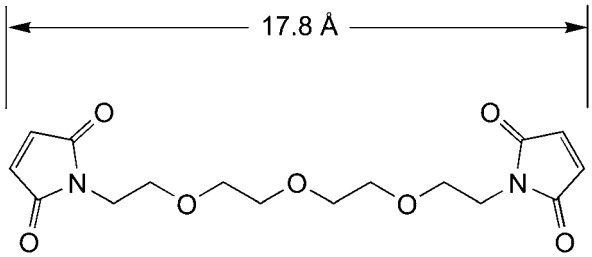


Figure 18.5 The modification of an APTS-modified surface containing amines with bis-NHS–PEG_5 yields hydrophilic spacers containing terminal NHS esters for coupling proteins.

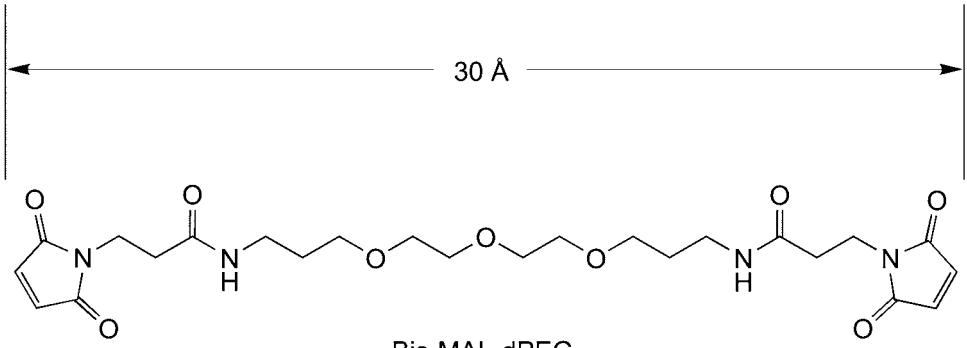
for poly(ethylene oxide), but to maintain a consistent nomenclature throughout this book, it is referred to under the PEG name. Another pair of crosslinkers, $BM(PEG)_3$ and bis-MAL–$dPEG_3$ have one additional PEG unit and provide similar bifunctional maleimides, but differ only in their structures leading from the PEG spacer to the maleimides (Figure 18.6). $BM(PEG)_5$,



$BM[PEG]_2$;
1,8-bis-Maleimidotriethyleneglycol
MW 308.29



$BM[PEG]_3$;
1,11-bis-Maleimidotriethyleneglycol
MW 352.34



Bis-MAL-$dPEG_3$
MW 522.55

Figure 18.6 The structures of $BM(PEG)_2$, $BM(PEG)_3$, and bis-MAL–$dPEG_3$ contain thiol-reactive maleimide groups on the ends of a discrete PEG spacer arm.

has a 17.8 Å spacer, while bis-MAL–dPEG$_3$ has a longer 30 Å length. These dimensions are measured between the outer hydrogens on the maleimide groups using linear three-dimensional structures after energy minimization. The actual structural configurations in aqueous solution almost certainly will not be linear and thus the molecular dimensions will differ from these values.

All of these bifunctional maleimides can be used to conjugate together two proteins containing available thiol groups (Figure 18.7). For instance, artificial bispecific antibodies have been created by coupling two disulfide-reduced antibodies together having different antigenic specificities using the thiol-reactive crosslinker SPDP (Chapter 5, Section 1.1). This was done by reducing the disulfides in the hinge region of antibodies to produce two heavy/light chain complexes containing one antigen binding site each and then conjugating the fragments from both antibodies together with SPDP (Foglesong *et al.*, 1989). Coupling one reduced antibody with another of a different specificity using a hydrophilic reagent such as BM(PEG)$_2$ will form a conjugate having two different antigen binding capabilities. The advantage of using a PEG-based compound over SPDP is greater water solubility of the resultant conjugate.

BM(PEG)$_2$ also can be used to determine protein–protein interactions or subunit interactions if there are available free thiol groups on each protein or subunit. The following protocol can be used to crosslink two thiol-containing proteins.

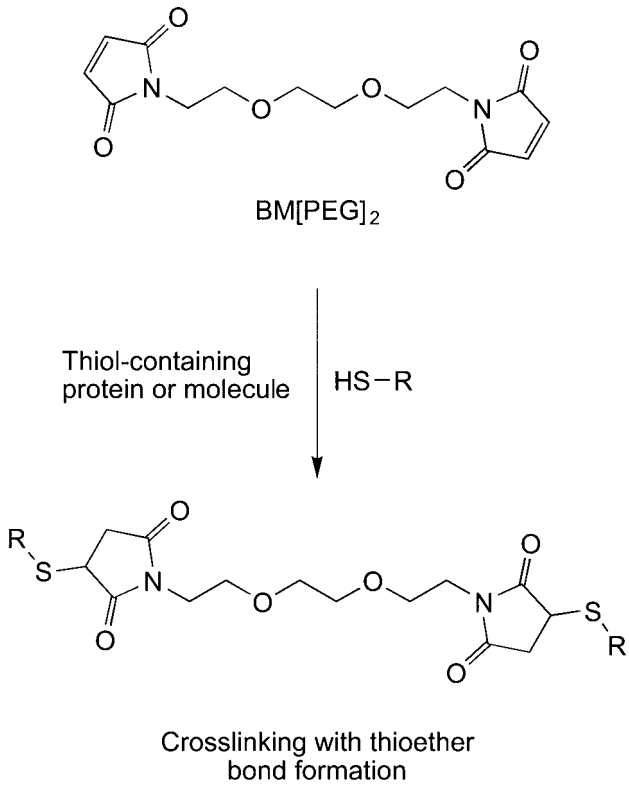


Figure 18.7 BM(PEG)$_2$ reaction with thiol-containing proteins forms crosslinks via thioether linkages.

Protocol

1. Dissolve the thiol-containing proteins to be crosslinked in 50 mM sodium phosphate, pH 6.5–7.5, containing 10 mM EDTA to prevent metal-catalyzed sulfhydryl oxidation.
2. Dissolve $BM(PEG)_2$ in DMSO or DMF at a concentration of 10–20 mM (3.1 mg/ml to make a 10 mM solution).
3. Add a quantity of the crosslinker solution to the protein solution to provide a 1- to 10-fold molar excess of crosslinker to protein. Lower molar ratios will help prevent oligomerization of protein if the proteins contain more than one available thiol group. A series of different molar ratios may be studied to optimize the level of reagent addition.
4. React for 1–2 hours at room temperature.
5. Purify the conjugate by dialysis or gel filtration.

2. Heterobifunctional PEG Reagents

Heterobifunctional crosslinkers contain different reactive groups or functionalities at each end of a spacer arm. Traditional reagents of this type are discussed in Chapter 5, but the PEG-based compounds are reviewed exclusively here, because of their unique water-soluble characteristics. The most popular aliphatic heterobifunctional compound is SMCC (or sulfo-SMCC), which contains an NHS ester on one end and a maleimide group on the other end. However, this compound suffers from a cross-bridge that is both water-insoluble and immunogenic. Redesigning this crosslinker to have a PEG cross-bridge provides enhanced water solubility for modified proteins or other molecules as well as displaying very low immunogenicity. These benefits of discrete PEG spacers create a new generation of heterobifunctional compounds, which dramatically improve the performance of conjugates made from them. The following sections describe these reagents in more detail.

2.1. Maleimide–PEG_n–NHS Ester Compounds

One of the most useful types of crosslinkers ever invented is a heterobifunctional compound containing an NHS ester on one end and a maleimide group on the other end. The ability to link an amine-containing molecule to another molecule containing a thiol group provides control over the conjugation process, which avoids the potential for oligomerization that often occurs when using homobifunctional crosslinkers. Probably the most significant recent development in this type of reagent is the introduction of PEG spacers in their cross-bridge construction. Unlike the previous iteration of these compounds that all used hydrophobic spacers (Chapter 5, Section 1), the PEG-based reagents provide water solubility both of the initial compound and of the crosslink itself after the reaction has taken place.

NHS–PEG_n–maleimide crosslinkers now are available as a series of different chain lengths of PEG within the cross-bridge, including repeating ethylene oxide units of 2, 4, 6, 8, 12, and 24 (Quanta BioDesign and Thermo Fisher). This series provides a range of molecular lengths after conjugation from 17.6 to 95.2 Å, so that an optimal size can be determined for nearly any application (Figure 18.8). The longest crosslinker of the group, NHS–PEG_{24}–maleimide, has a total length that compares to almost the diameter of a typical immunoglobulin (IgG) antibody molecule. Longer chain NHS–PEG–maleimide crosslinkers may be appropriate especially for

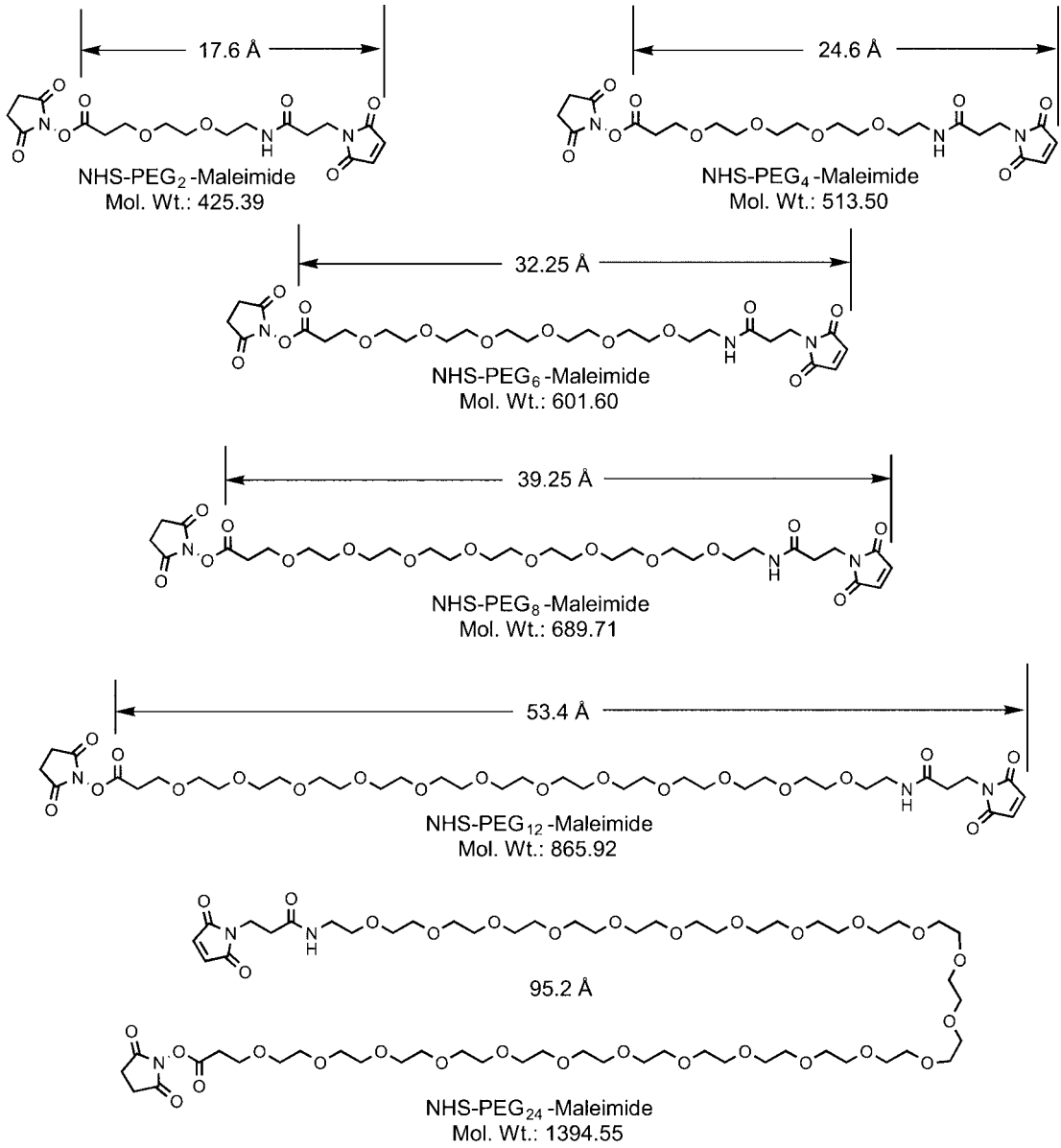


Figure 18.8 Series of NHS–PEG–maleimide crosslinkers.

the masking of surfaces to permit coupling of affinity ligands, while avoiding the potential for nonspecific interactions with the surface structure. In addition, these heterobifunctional PEG compounds are superior choices for linking haptens to carrier proteins to create immunogens. In this application, the PEG chain provides a high degree of freedom of motion and does not illicit an immune response itself, thus effectively generating antibodies *in vivo* with greater specificity toward the hapten, not the carrier or the crosslinker.

In use, NHS–PEG–maleimides first are reacted in at least a 10-fold molar excess with an amine-containing molecule, such as a protein, to form an intermediate derivative with terminal maleimide groups. Depending on the number of maleimide modifications needed, the molar excess of crosslinker may be adjusted to provide the desired level of activation. The maleimide groups are more stable in aqueous solution than the NHS esters, so the modified protein can be purified from excess crosslinker before reacting it with a sulfhydryl-containing molecule or protein. After purification, the maleimide–PEG–protein derivative is reacted with a second protein containing sulfhydryls to form the final conjugate via a thioether bond (Figure 18.9). The higher the number of maleimide–PEG modifications on the first protein, the greater the number of potential sulfhydryl-containing proteins may be conjugated to it. The ratio of the reactants is often chosen to give at least several thiol–proteins conjugated to each of the maleimide–PEG–protein derivatives. Optimization of this ratio should be done to determine the best conjugate for a given application.

The intermediate maleimide–PEG–protein derivative maintains excellent solubility due to the presence of the PEG chains. Unlike a hydrophobic NHS–maleimide-type crosslinker made from an aliphatic cross-bridge, the PEG chains have a tendency to increase the solubility of modified proteins or other molecules. This effect is amplified when using PEG compounds of longer dimensions. The result is that modified proteins remain soluble, and even if not every maleimide group gets conjugated to a thiol-containing molecule, the presence of unreacted crosslinkers on the protein surface doesn't contribute to nonspecific interactions of the conjugates.

Most of these PEG crosslinkers come as thick, sticky, viscous liquids or low melting solids (PEG_2). For this reason, weighing out a small sample of a compound can be difficult or impossible. It usually is best to dissolve an entire vial or a larger amount in organic solvent at a known concentration to permit accurate dispensing of a smaller amount into a reaction. Suitable solvents to prepare a stock solution include dry (molecular sieved) DMSO, DMF, DMAC, acetonitrile, or methylene chloride (for non-water-miscible reactions).

The following protocol is a general guide for using NHS–PEG_n–maleimide crosslinkers. This method may be used as a starting point for developing an optimized procedure for creating a unique conjugate.

Protocol

1. In a fume hood, dissolve the NHS–PEG_n–maleimide compound of choice in a dry, water-miscible organic solvent to make a concentrated stock solution. For instance, to prepare a 100 mM solution of the NHS–PEG_6–maleimide reagent (MW 601.6), dissolve an entire 100 mg vial of the crosslinker in 1.66 ml of DMAC (dry DMSO or DMF work well, too).
2. Dissolve the amine-containing protein to be activated in 0.1 M sodium phosphate, pH 7.2 (coupling buffer). A protein concentration of 1–10 mg/ml in buffer will work well in this protocol. For more dilute protein solutions, greater quantities of the NHS–PEG_n–maleimide compound may have to be added to obtain equivalent levels of modification.
3. Add a quantity of the crosslinker solution to the protein solution to provide at least a 10-fold molar excess of reagent over the amount of protein present. Higher levels of reagent-to-protein may be used, even up to a 50-fold molar excess, to obtain a large number of active groups for subsequent coupling to a thiol–protein. Conversely, the use of lower molar ratios will limit the number of maleimide–PEG modifications on each

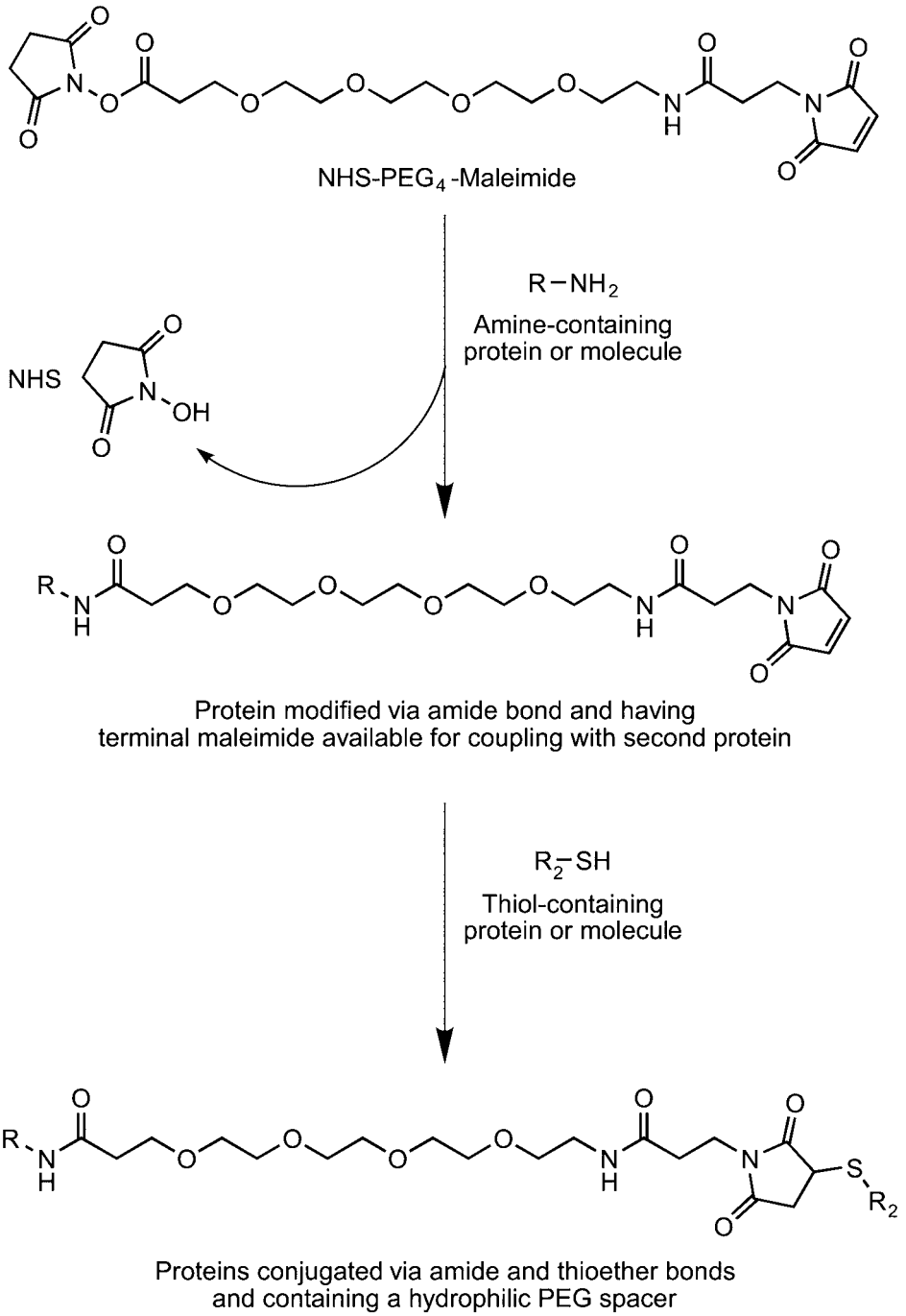


Figure 18.9 NHS–PEG_4–maleimide conjugation reactions are carried out in two steps involving modification of an amine-containing molecule with the NHS ester end with subsequent coupling of the maleimide end with a thiol-containing molecule.

protein to just a few. A series of reactions using different concentrations of crosslinker may have to be done to determine the optimal level to use for a particular application.
4. React with gentle mixing for 30 minutes at room temperature or 60 minutes at 4°C.
5. Remove excess crosslinker by centrifugal dialysis or gel filtration using a molecular weight exclusion of 5,000. This procedure should be done quickly due to the labile nature of the maleimide group in aqueous solution, which will hydrolyze to a ring-open maleamic acid that is ineffective at coupling to thiols.
6. Dissolve a sulfhydryl-containing protein in coupling buffer containing 10 mM EDTA at a concentration of at least 1–10 mg/ml. Thiols may be generated in proteins by disulfide reduction or through use of a thiolation reagent (Chapter 1, Section 4.1). The amount of the thiol–protein needed is determined by the optimal molar ratio desired in the final conjugate of the thiol-protein to the maleimide-activated protein purified in Step 5. Often, this means doing a reaction of the thiol–protein to the maleimide–PEG–protein in at least a 4:1 molar ratio, but higher ratios (e.g., 15:1) also can be used, depending on the application. Note that the number of thiol–protein molecules that can be conjugated with the maleimide–PEG–protein is limited by the number of reactive maleimide groups present and also by the molecular size of the two molecules being conjugated. Steric crowding will limit how many thiol–proteins can be attached to the maleimide-activated protein.
7. Add the thiol–protein to the maleimide–PEG–protein in the desired molar ratio to initiate the conjugation reaction.
8. React with gentle mixing for at least 2 hours at room temperature or overnight at 4°C.
9. The conjugate may be purified to remove unconjugated protein using gel filtration on a column of resin having a molecular weight cut-off able to accommodate the proteins being separated.

2.2. NHS-PEG$_n$-Azide/Alkyne Compounds for Chemoselective Ligation

Another family of heterobifunctional compounds containing an internal PEG spacer is reagents with an NHS ester on one end and either an azide or alkyne group on the other end (Figure 18.10). These reagents react with amines on proteins or other molecules to create an amide bond derivative having a terminal functionality suitable for use in chemoselective ligation reactions, particularly click chemistry cycloaddition and Staudinger ligation (Chapter 17, Sections 4 and 5). Click chemistry is the reaction between an azide and an acetylene group in the presence of a catalytic amount of Cu(I) yielding a triazole ring linkage, which is a very efficient process for bioconjugation purposes. NHS ester heterobifunctional compounds containing these groups may be used to functionalize biomolecules, organic compounds, particles, or surfaces with azides or alkynes. Since both the azido and acetylene groups are highly stable in aqueous solution or in the presence of biomolecules, the modified substances or devices maintain nearly indefinitely the ability to conjugate molecules containing the opposite functionality.

The azide-containing PEG compounds also can be used in the Staudinger ligation process with a phosphine-containing group having an electrophilic trap. This reaction, like the click chemistry process, is chemoselective and highly bioorthogonal in that the functional groups won't react with typical functionalities present in biological solutions. The Staudinger reaction with PEG-azide compounds proceeds to give an amide bond with either retention of the

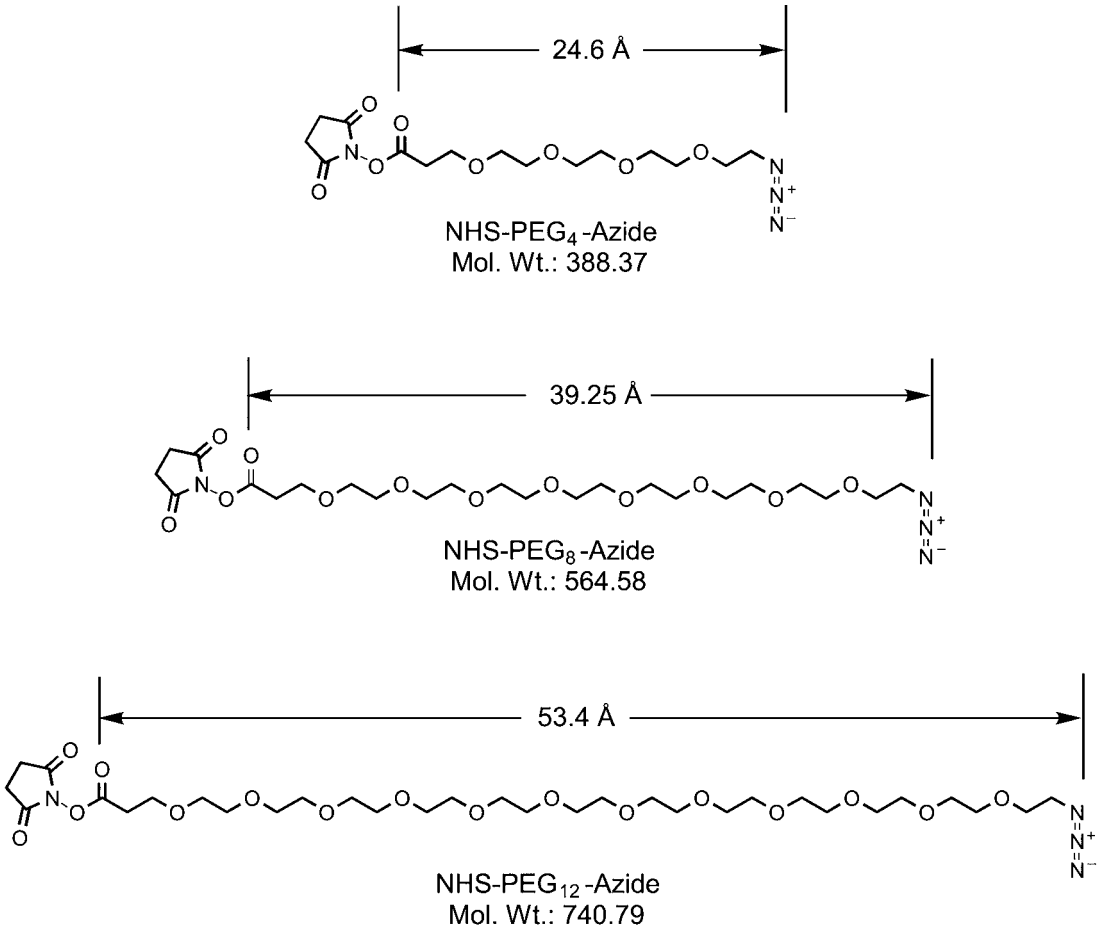


Figure 18.10 NHS-PEG-azide compounds can be used to modify amine-containing proteins or other molecules for subsequent conjugation using either the click chemistry reaction or Staudinger ligation.

phosphine component or, in the traceless version of the reaction, with loss of the phosphine and formation of a zero-length crosslink between the two reacting molecules (Figure 18.11).

Click chemistry or Staudinger ligation equipped heterobifunctional crosslinkers containing a hydrophilic PEG spacer provide a stable, yet highly efficient means to conjugate or immobilize biomolecules. The PEG spacer provides water solubility, low nonspecific binding character, and low immunogenicity.

Three PEG lengths are available in NHS-PEG$_n$-azide compounds, including $n = 4$, 8, or 12 ethylene oxide repeat units (Figure 18.10) (Quanta BioDesign). The NHS-PEG$_4$-azide compound provides a 16-atom spacer, which is 17.7 Å long after reaction with an amine-containing molecule. The NHS-PEG$_8$-azide and the NHS-PEG$_{12}$-azide reagents provide longer spacer arms having molecular distances of 32.2 Å and 46.4 Å, respectively. All of these measurements are done using three-dimensional molecular models that are linearized structures with distances taken from the NHS ester carbonyl group to the first nitrogen atom of the azide. The actual

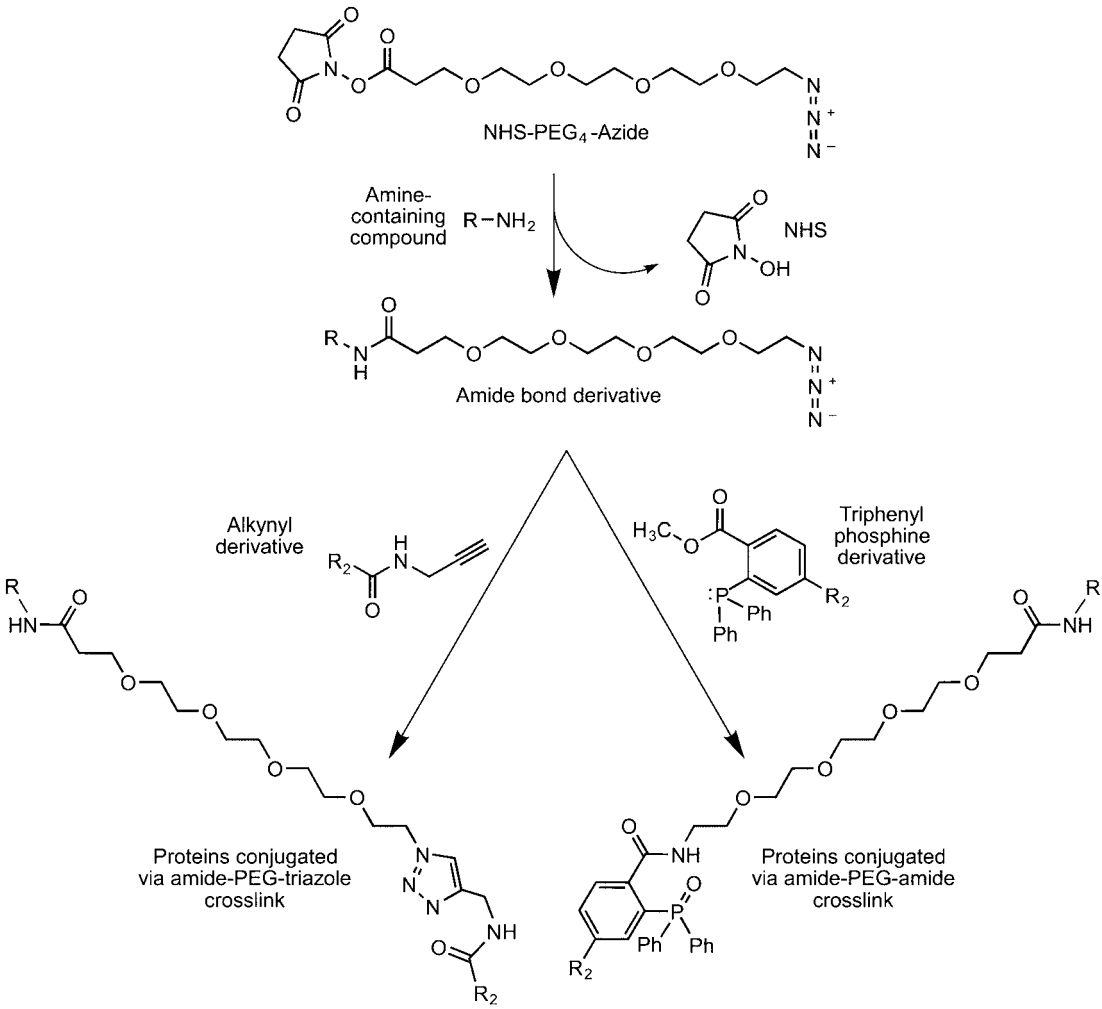


Figure 18.11 NHS-PEG_4-azide can be used to modify an amine-containing molecule to create an amide derivative terminating in azido groups. The azide modifications then can be used in a click chemistry reaction that forms a triazole linkage with an alkyne-containing molecule. Alternatively, the azide derivative can be used in a Staudinger ligation reaction with a phosphine derivative, which results in an amide bond linkage.

molecular dimensions between crosslinked molecules will differ from these values due to the different conformations that the PEG chains can take in aqueous solution and due to the formation of the triazole ring after the click reaction has taken place.

Only one compound is currently available for modifying amine-containing molecules with an acetylene group, which is the propargyl–PEG_1–NHS ester containing only a single ethylene oxide unit (Figure 18.12) (Quanta BioDesign). Other simple acetylene-based raw materials also are commercially available with functional groups, such as propargylamine and various carboxylic acid derivatives (4-pentynoic acid, Aldrich), which can be used in a carbodiimide reaction to modify molecules or surfaces. However, at the time of this writing, no longer chain

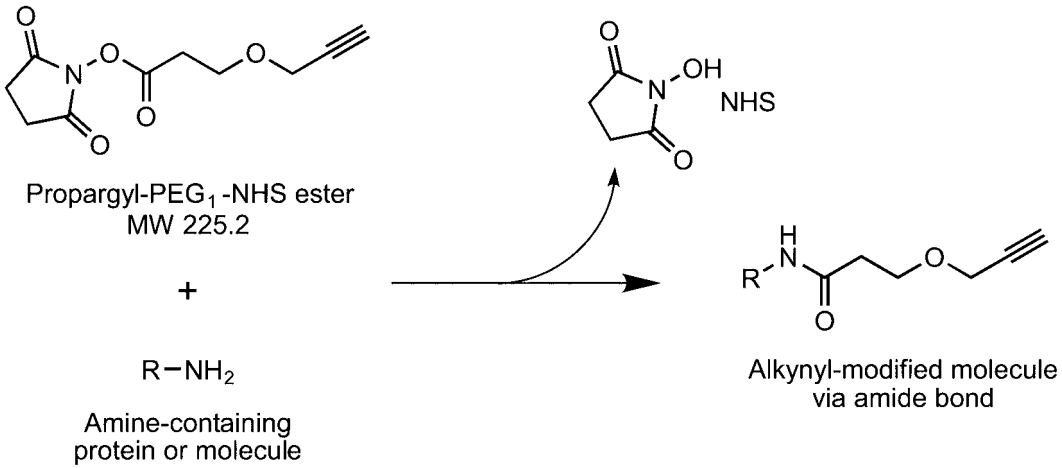


Figure 18.12 Propargyl–PEG_1–NHS ester can be used to react with amine-containing molecules to add short alkyne modification sites for subsequent click chemistry reactions with azide-containing molecules.

PEG-based reagents are available with the terminal alkyne needed for a click chemistry bioconjugation reaction.

The following protocol may be used to modify a protein for coupling to a surface using the NHS-PEG_n-azide and propargyl–PEG_1–NHS ester compounds. Similar procedures may be used to conjugate two molecules together, such as forming an antibody–enzyme conjugate.

Protocol

Modification of protein 1 with NHS-PEG_n-azide groups

1. Dissolve a first protein to be modified with an azide group in 100 mM sodium phosphate, pH 7.2 (PBS), at a concentration of at least 1–10 mg/ml.
2. In a fume hood, prepare a stock solution of an NHS-PEG_n-azide by dissolving it at a concentration of 20 mM in DMAC, DMSO, or DMF (using highly pure and dry solvent).
3. Add a quantity of the crosslinker solution to the protein solution with mixing to provide at least a 10-fold molar excess of crosslinker over the amount of protein present.
4. React with mixing for 30–60 minutes at room temperature.
5. Purify the azido-PEG–protein by dialysis or gel filtration using PBS to remove excess reactants and solvent. The azide modifications are stable to aqueous conditions, so the protein derivative may be stored in this form until needed, provided the protein is stable.

Modification of protein 2 with propargyl–PEG_1–NHS ester

1. Dissolve a second protein to be functionalized with alkyne groups at a concentration of 1–10 mg/ml in PBS.
2. In a fume hood, prepare a stock solution of propargyl–PEG_1–NHS ester in DMAC, DMSO, or DMF (highly pure and dry) at a concentration of 20 mM (4.5 mg/ml).
3. Add a quantity of the propargyl–PEG_1–NHS ester solution with stirring to the protein solution to obtain a 10-fold molar excess. Optimization of reactant ratios may be done to determine the best modification for a particular conjugation.

4. Gently mix for 30–60 minutes at room temperature.
5. Purify the alkyne-modified protein from excess linker by dialysis or gel filtration. The acetylene modification is stable in aqueous solution at this point.

Coupling the alkyne-protein to the azide-protein

1. With mixing, add a quantity of the azide-modified protein to the alkyne-modified protein to provide the desired molar excess over the quantity of protein and amount of alkyne groups present. Maintain the overall protein concentration at 1–10 mg/ml or greater to obtain the best reaction kinetics. Often, a one protein is reacted in 4- to 15-fold molar excess over a second protein to create a conjugate having several molecules of the first protein attached to the second protein. However, the appropriate molar excess may have to be determined by doing a series of reactions at different ratios and determining the best ratio for use in a particular application.
2. Add 2.5 μl of a solution containing 10 mM $CuSO_4 \cdot 5H_2O$ and 50 mM ascorbic acid dissolved in water per ml of the protein mixture. Mix to dissolve.
3. React with mixing at room temperature for at least 4 hours (or at 4°C overnight, if the protein is not stable at ambient temperature). The optimal time of reaction is dependent on the proteins being coupled together and the number of azide and alkyne reactive groups available on them. An alkynyl-protein added to the azide-protein at a high molar ratio probably would reach maximal coupling yield in a matter of hours.
4. Purify the protein conjugate by dialysis or gel filtration using a molecular weight cut-off appropriate for the sizes of the proteins being separated.

A similar protocol may be used to couple proteins or molecules modified with an azide or alkyne to a particle or surface modified with the other functionality.

3. Biotinylation Reagents Containing Discrete PEG Linkers

Biotin modification reagents are widely used to attach a biotin group to proteins or other molecules for subsequent use in avidin, streptavidin, or NeutrAvidin separations or assays. Traditional biotin compounds containing aliphatic or other hydrophobic linker arms are discussed in detail in Chapter 11. In this section, the biotin–PEG compounds exclusively are discussed due to their unique hydrophilic properties, which include low nonspecific binding character and low immunogenicity.

Traditional biotinylation compounds include the very popular (sulfo)NHS-LC-biotin, which contains either an uncharged NHS ester or a negatively charged sulfo-NHS ester in addition to a C_6 alkyl chain leading to the biotin group. Although the sulfo-NHS ester provides a degree of water solubility for the entire compound prior to using it to modify a protein, the resultant LC-biotin modification left on the protein is extremely hydrophobic. The result of adding such hydrophobic groups to a protein surface often is a balance between maintaining water solubility or tending toward protein aggregation and precipitation. Many antibodies that are biotinylated with NHS-LC-biotin undergo slow aggregation and loss of activity despite limiting the degree of biotinylation during production.

The use of discrete PEG spacers in the construction of biotinylation compounds not only increases the water solubility of the modification reagent itself, but significantly increases the hydrophilicity and stability of proteins modified with them. Even when high modification levels

are used with PEG–biotin compounds, the resultant biotinylated protein typically is very water soluble and does not aggregate in solution.

The following compounds represent some of those that are commercially available for adding a PEG–biotin group to proteins and other molecules. Reactive group options include NHS esters for coupling to amines, maleimide groups for coupling to thiols, hydrazides for conjugation with carbonyl compounds, and a photoreactive benzophenone for nonselective insertion into molecular structures (Thermo Fisher, Quanta BioDesign, Solulink, and Molecular Biosciences). In addition, a chromogenic biotin compound containing a PEG spacer is available from Thermo Fisher and Solulink. This unique compound allows exact measurement of the amount of biotinylation by measuring the absorbance of the final purified conjugate.

3.1. NHS–PEG$_n$–Biotin Compounds

NHS ester biotinylation reagents are the most popular choice for adding a biotin group to another molecule that contains an available amine. The ester reacts with an amine at neutral or slightly alkaline pH values to form a stable amide bond with the amine-containing protein or molecule. Discrete PEG biotinylation compounds containing an NHS ester are available in chain lengths of 4 or 12 repeating polyethylene oxide units and in one form that contains a cleavable disulfide in the cross-bridge.

The NHS ester compounds are sensitive to hydrolysis in aqueous solution, and they likely will hydrolyze faster than more hydrophobic biotinylation compounds due to their hydrophilicity. If a stock solution is made at a higher concentration to facilitate the addition of a small amount to a reaction solution, the initial solution should be made in a water-miscible organic solvent that is dried with a molecular sieve. Suitable solvents include DMAC, DMSO, or DMF. If using DMF, use only highly pure solvent, as it may contain amines that can react with the NHS ester groups (Figure 18.13).

Reactions done with NHS–PEG$_n$–biotin compounds typically are done with the reagent in molar excess over the amount of protein being modified. The efficiency of the reaction is dependent on the concentrations of reactants and the solvent exposed area of the amine groups on the protein. Reactions done with a 10-fold molar excess of NHS–PEG$_n$–biotin usually will result in at least 2–3 biotin labels per protein, while doubling the molar excess should provide 4–6 biotinylations. The optimal number of biotin groups added to a particular protein should be determined experimentally to provide the best performance in the intended application.

NHS-SS-PEG$_4$-biotin contains a cleavable disulfide bridge next to the PEG chain. This feature allows the biotin group to be released from the biotinylated molecule using a disulfide reducing agent, such as DTT or TCEP (Figure 18.14). This is useful for doing immunoprecipitation (IP) or co-immunoprecipitation (co-IP) assays, because a biotinylated antibody can be used to capture and affinity isolate a target protein on immobilized (strept)avidin and then elute the bound proteins by reduction (using 50 mM DTT for 2 hours at room temperature or for 30 minutes at 50°C). This eliminates the severe denaturing conditions usually required to break the (strept)avidin–biotin bond, thus better preserving the native structure and activity of the captured proteins. Compounds of this type also may be used successfully in cell-surface biotinylation, as described in Chapter 11, Section 1, under sulfo-NHS-SS-biotin.

The following protocol is based on the applications done at Thermo Fisher (formerly Pierce). The use of higher pH values for the NHS ester reaction than those recommended may result

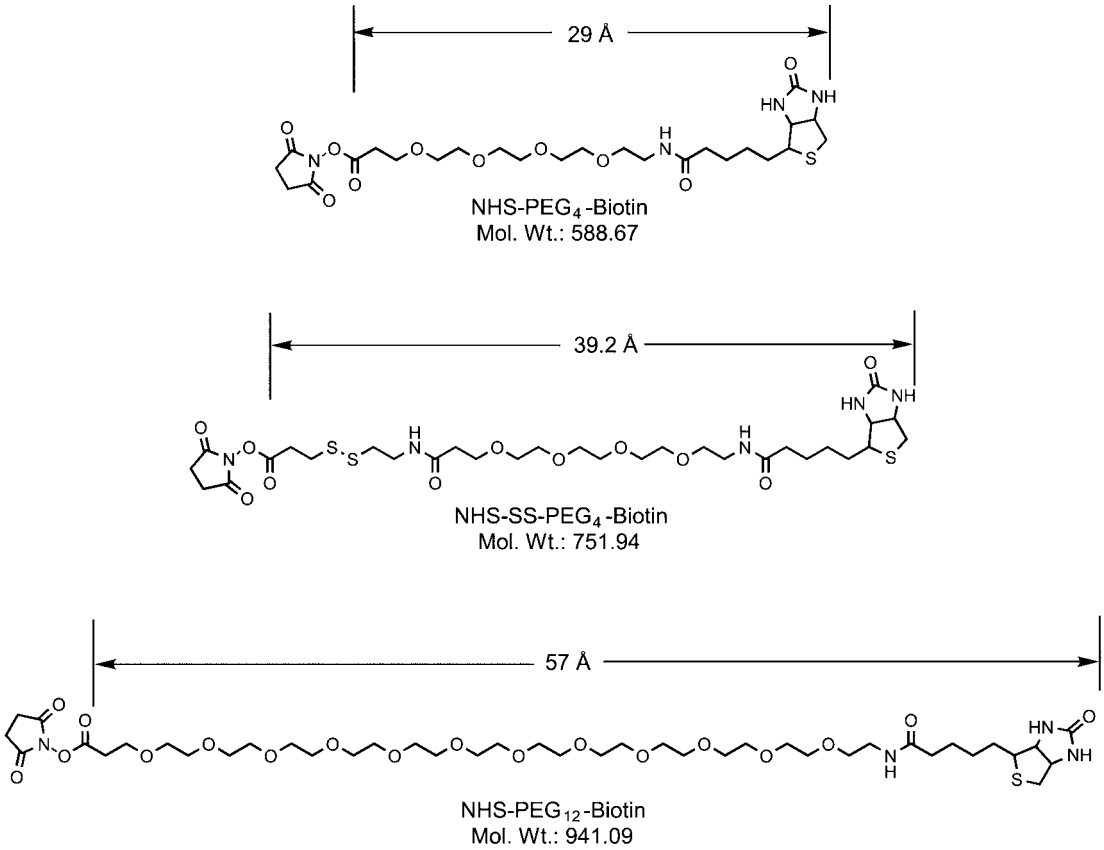


Figure 18.13 NHS–PEG$_n$–biotin compounds.

in lower biotinylation yields due to increased hydrolysis, especially when using an extremely hydrophilic PEG compound.

Protocol

1. Dissolve an antibody or protein to be modified at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5. Lower concentrations of protein may result in decreased reaction yields and require increased quantities of reagent to obtain acceptable levels of biotinylation. Avoid amine-containing buffers or components, such as Tris or imidazole, which will react with the NHS ester and interfere with the biotinylation process.
2. Dissolve the NHS–PEG$_n$–biotin compound in DMAC, DMSO, or DMF (pure and dry) at a concentration of 10–20 mM. Prepare fresh in a fume hood.
3. With mixing, add an aliquot of the biotinylation stock solution to the protein solution to provide at least a 10-fold molar excess over the concentration of protein present. Doing a series of reactions with different molar amounts of the NHS–PEG$_n$–biotin compound may be done to optimize the modification level.
4. React with gentle mixing for 30–60 minutes at room temperature or 2 hours at 4°C.

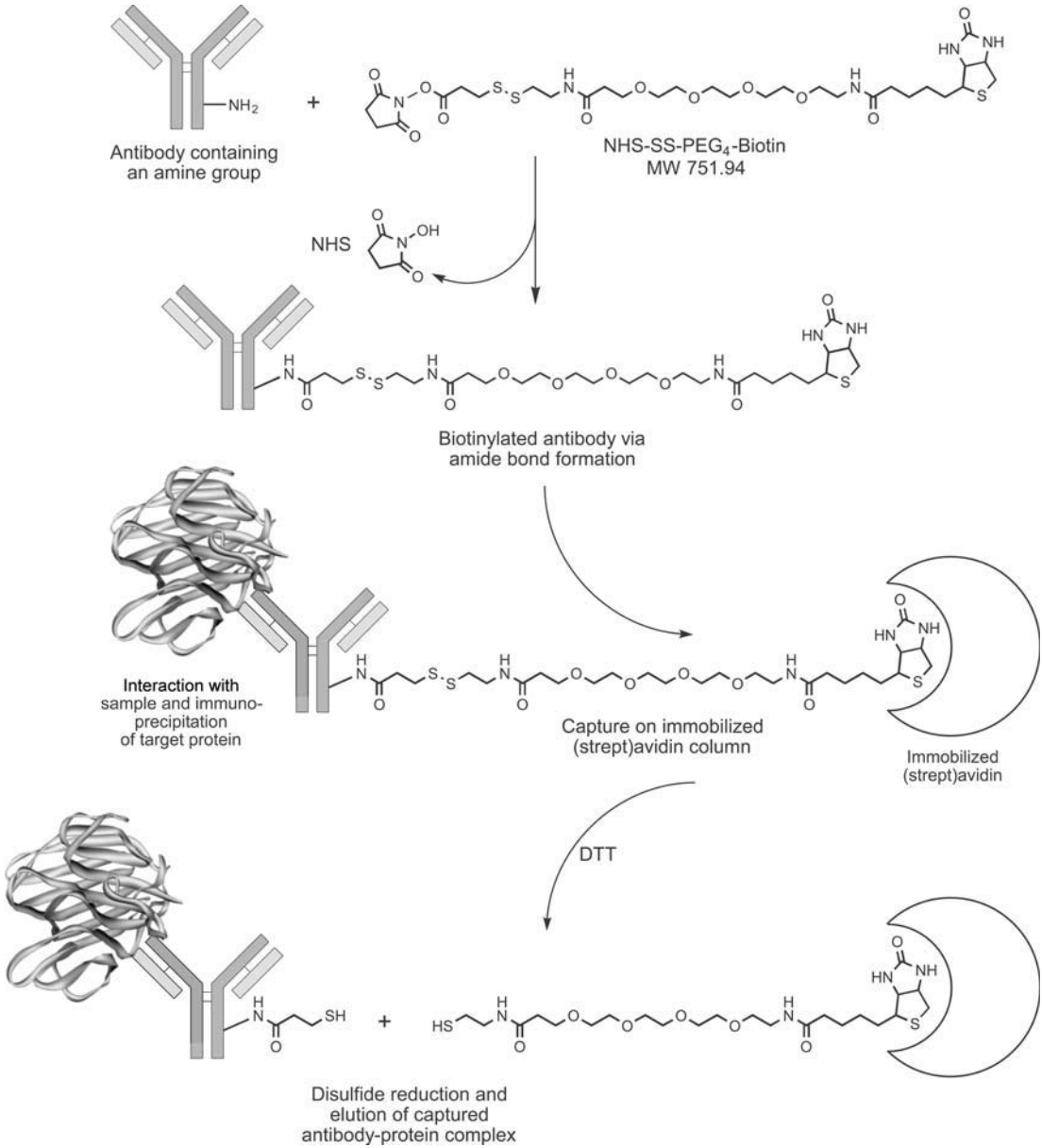


Figure 18.14 NHS-SS-PEG$_4$-biotin can be used to label a primary antibody molecule that has specificity for a protein or interest. Incubation of the biotinylated antibody with a sample, such as a cell lysate, allows the antibody to bind to its target. Capture of the antibody–antigen complex on an immobilized streptavidin reagent effectively isolates the targeted protein from the other proteins in the sample. The disulfide linkage in the spacer arm of the biotin tag permits elution of the immune complex from the streptavidin support using DTT and without using the strong denaturing condition typically required to break the streptavidin–biotin interaction.

5. Purify the biotinylated protein from excess reagent and reaction by-products using dialysis or gel filtration (desalting resin).

3.2. NHS-Chromogenic-PEG_3-Biotin

A novel detectable biotinylation reagent containing a hydrophilic PEG spacer is NHS-chromogenic-PEG_3-biotin (also called chromogenic biotin; Thermo Fisher, Solulink; Figure 18.15). Next to the terminal NHS ester of this compound is a bis-aryl hydrazone group created from the reaction of a 6-hydrazinium nicotinate derivative and a benzaldehyde group to form the chromogen having an absorbance at 354 nm (ε = 29,000 $M^{-1}cm^{-1}$). The NHS ester end can be used to modify amine-containing molecules and form a stable amide linkage (Figure 18.16). The spacer arm contains a hydrophilic spacer made from three ethylene oxide units, which provide water solubility for the compound.

Proteins biotinylated with this reagent will have a characteristic absorbance band at 354 nm, which can be used to determine accurately the number of biotin groups per molecule. No other biotinylation compound has such built-in quantification capability. This feature eliminates the need to consume conjugate by doing a HABA assay to test for the level of biotin incorporation (Chapter 23, Section 7).

The following protocol is adapted from the manufacturers' recommendations.

Protocol

1. Dissolve a protein or other amine-containing molecule to be biotinylated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5, at a concentration of 1–10 mg/ml. Note

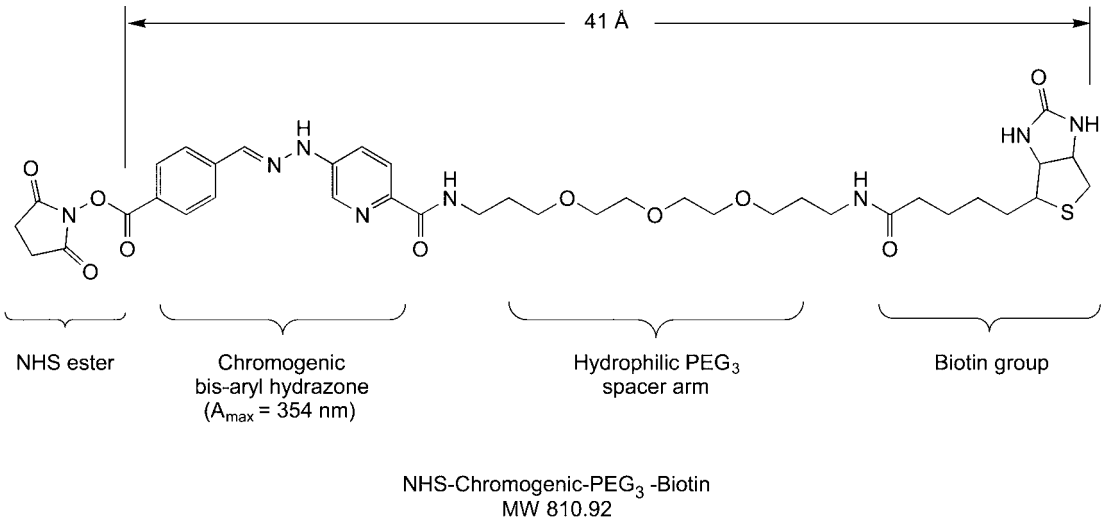


Figure 18.15 NHS-chromogenic-PEG_3-biotin contains an amine-reactive NHS ester that can be used to label biomolecules through an amide linkage. The chromogenic bis-aryl hydrazone group within the spacer arm of the reagent allows the degree of biotinylation to be quantified by measuring its absorbance at 354 nm. The compound also contains a hydrophilic PEG spacer, which provides greater water solubility.

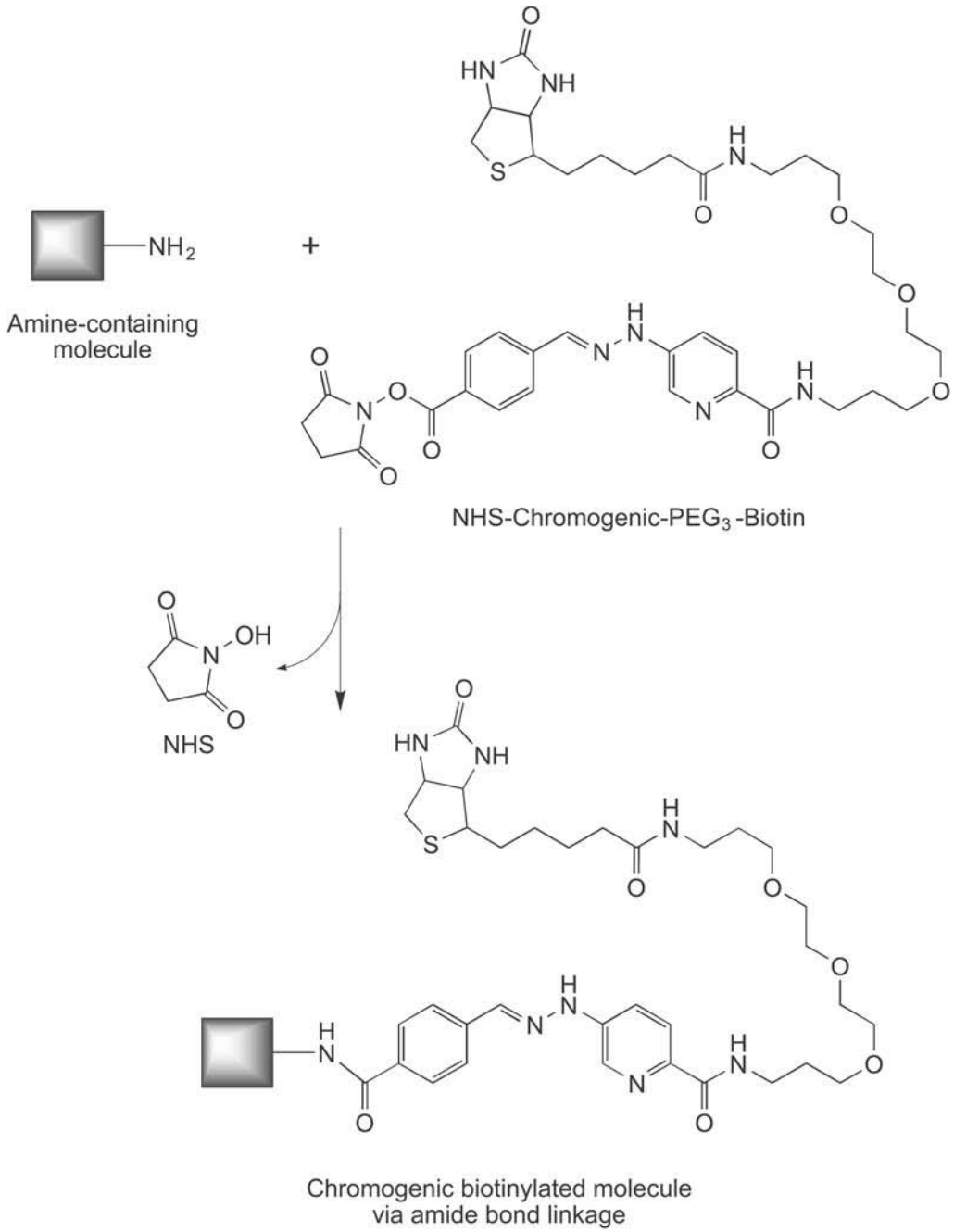


Figure 18.16 NHS-chromogenic-PEG_3-biotin reacts with amine groups in proteins or other molecules to form amide bond derivatives.

that protein solutions that are more dilute than this may require higher levels of biotinylation reagent addition to achieve the same yield of modification.
2. In a fume hood, dissolve NHS-chromogenic-PEG$_3$-biotin in DMF at a concentration of 12.33 mM (2 mg/200 µl DMF). With mixing, add a quantity of the reagent to the protein solution to provide the desired molar excess (i.e., 10- to -20 fold excess).
3. React for 30–60 minutes at room temperature or 2 hours at 4°C.
4. Purify the modified protein from unreacted biotinylation reagent and reaction by-products using dialysis or gel filtration. Complete removal of the excess reagent is necessary to provide accurate measurement of the biotin incorporation level by absorptivity.
5. Measure the absorbance of the biotinylated protein solution at 354 nm. Use the molar extinction coefficient for the chromogenic group (ε = 29,000 $M^{-1}cm^{-1}$) to determine the concentration of biotin present. To determine the molar ratio of biotin-to-protein, divide the molar concentration of biotin by the molar concentration of protein present (which may be determined by using the Coomassie assay or the BCA assay methods).

3.3. Maleimide–PEG$_n$–Biotin Compounds

Discrete PEG–biotin compounds containing a terminal maleimide group may be used to label sulfhydryl-containing proteins and other molecules through thioether bond formation (Figure 18.17). The targeting of thiol groups in proteins often is used to direct the modification reaction away from binding sites or active centers in proteins, thus preserving activity. Maleimide reagents in general are the second most-popular reactive group used for bioconjugation purposes, second only to NHS esters. Unlike biotinylation compounds containing a hydrophobic hydrocarbon chain (Chapter 11), the discrete maleimide–PEG-based reagents provide increased hydrophilicity for modified molecules and maintain solution stability even at high substitution levels.

The maleimide group reacts with thiols in the pH range of 6.5–7.5 to form a stable thioether linkage with very little cross-reactivity with amines at this pH (Figure 18.18). However, the maleimide ring is subject to hydrolysis in aqueous solution, and since it is next to an extremely hydrophilic PEG chain in these reagents, this factor may increase the hydrolysis rate beyond that typically observed with hydrocarbon-based spacers (Chapter 2, Section 2.2). For this reason, stock solutions of a maleimide–PEG$_n$–biotin compound should be made in highly pure and dry organic solvent, which then can be added to an aqueous reaction medium to commence the biotinylation process.

The three maleimide–PEG$_n$–biotin compounds illustrated in this section provide short, medium, and very long chain spacer options, with the longer chains resulting in the greatest degree of hydrophilicity of modified molecules. The following protocol is adapted from general maleimide-based biotinylation methods, as discussed in Chapter 11, Section 2.

Protocol

1. Dissolve a sulfhydryl-containing protein or other thiol-molecule in a thiol-free buffer within a pH range of 6.5–7.5. The use of 20 mM sodium phosphate, 150 mM NaCl, pH 7.2, works well for this reaction. The concentration of protein should be in the range of 1–10 mg/ml. Lower concentrations of protein may result in the need to increase the molar excess of biotinylation reagent to obtain an acceptable level of modification. If a

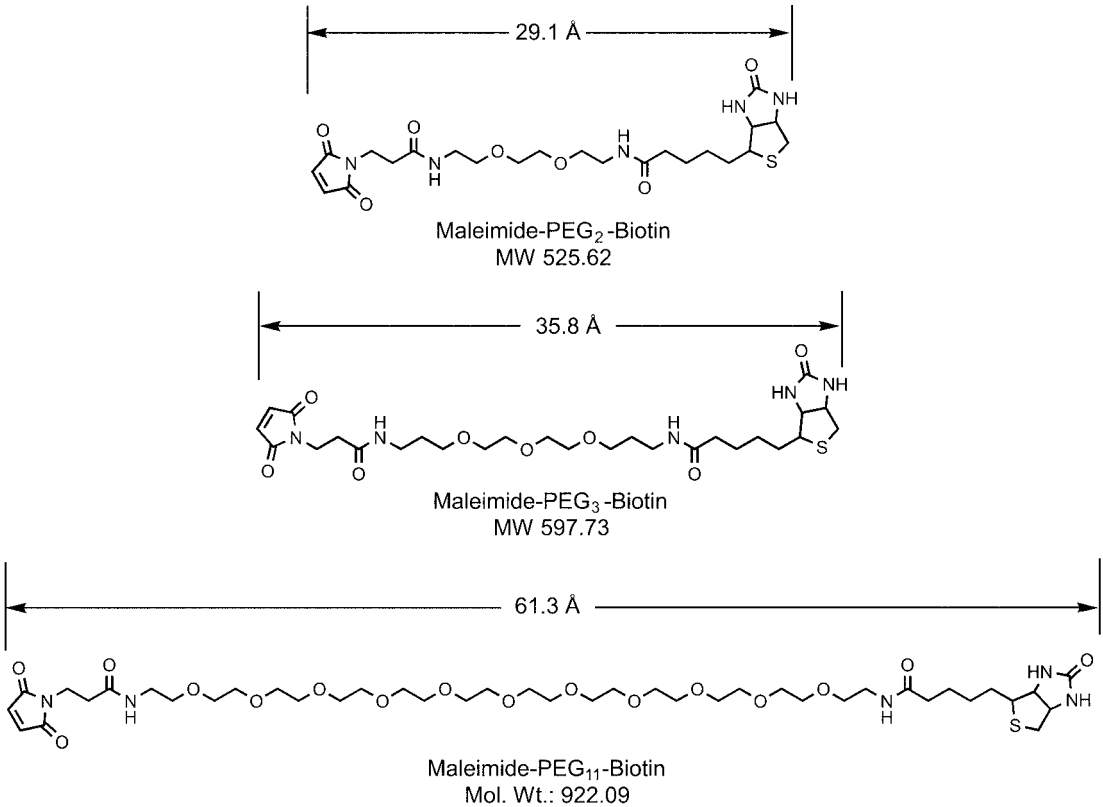


Figure 18.17 Maleimide–PEG$_n$–biotin compounds of three different discrete PEG sizes are available, including a PEG$_{11}$ chain that provides a molecular length of over 60 Å.

thiol is not present on the molecule to be biotinylated, one may be created by disulfide reduction or through the use of a thiolation reagent (Chapter 1, Section 4.1).

2. Prepare a stock solution of the maleimide–PEG$_n$–biotin compound in DMAC, DMSO, or DMF (pure and dry solvents only) at a concentration of 10–20 mM.
3. With mixing, add an aliquot of the biotin solution to the protein solution to obtain at least a 10-fold molar excess over the quantity of protein present. As thiols typically are present in limiting amounts on proteins, the use of a high-molar reagent ratio is not required to achieve acceptable yields of biotinylation.
4. React with gentle mixing for 2 hours at room temperature or 4 hours at 4°C.
5. Purify the biotinylated protein by dialysis or gel filtration using a desalting resin.

3.4. Hydrazide-PEG$_4$-Biotin

Hydrazide-containing PEG-biotinylation reagents provide reactivity with carbonyl groups (e.g., aldehydes) to label carbohydrates or glycoproteins via hydrazone bond formation (Figures 18.19 and 18.20). The hydrazide group also may be coupled with carboxylate-containing

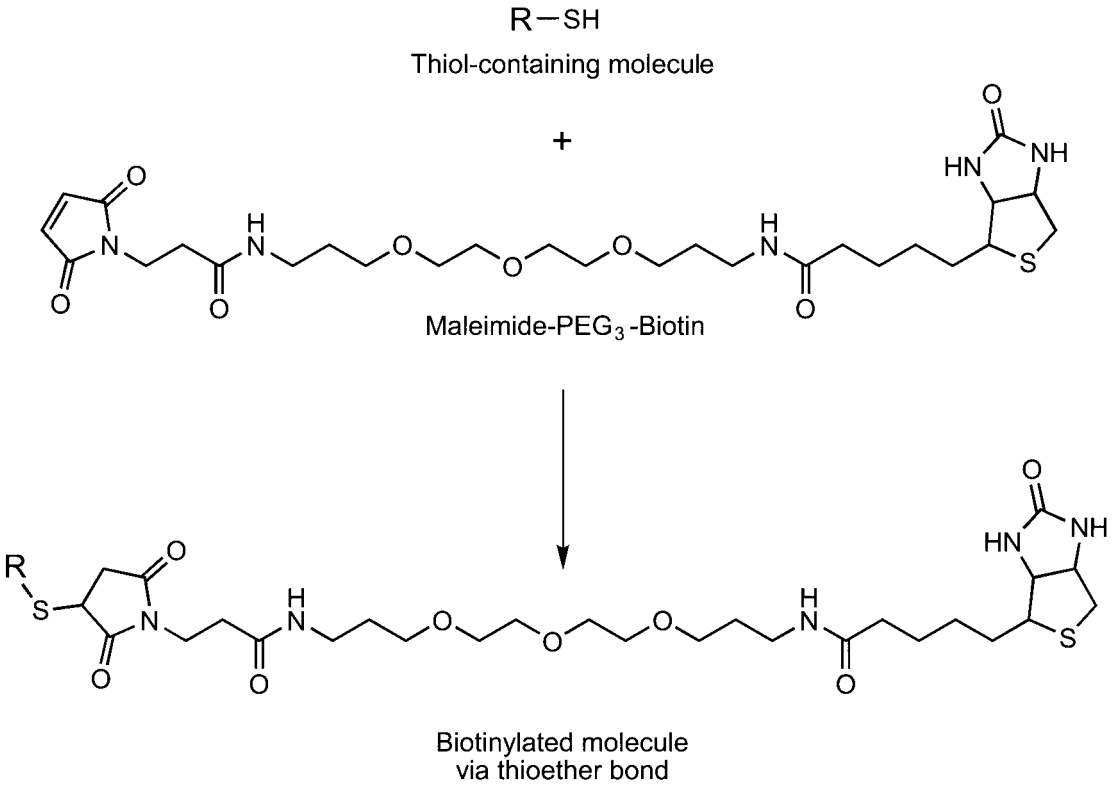


Figure 18.18 Maleimide–PEG$_n$–biotin compounds react with thiol-containing molecules to form thioether linkages.

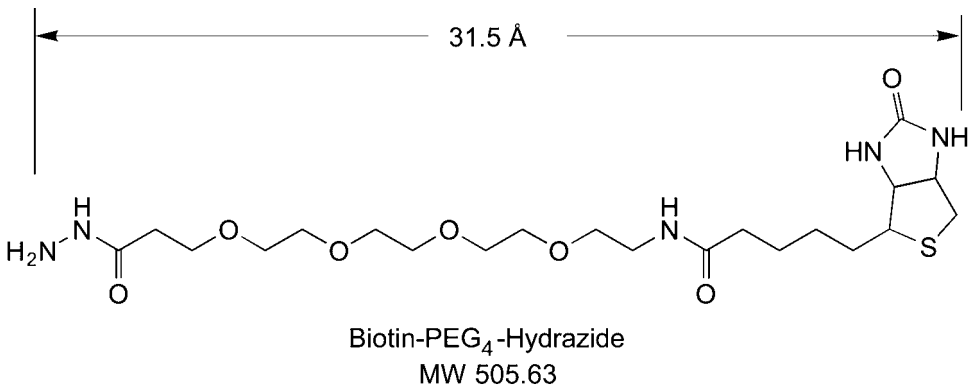


Figure 18.19 Biotin-PEG$_4$-hydrazide is a hydrophilic biotinylation reagent that can be used to modify glycans or carbohydrates at their reducing end or after periodate oxidation to create aldehydes.

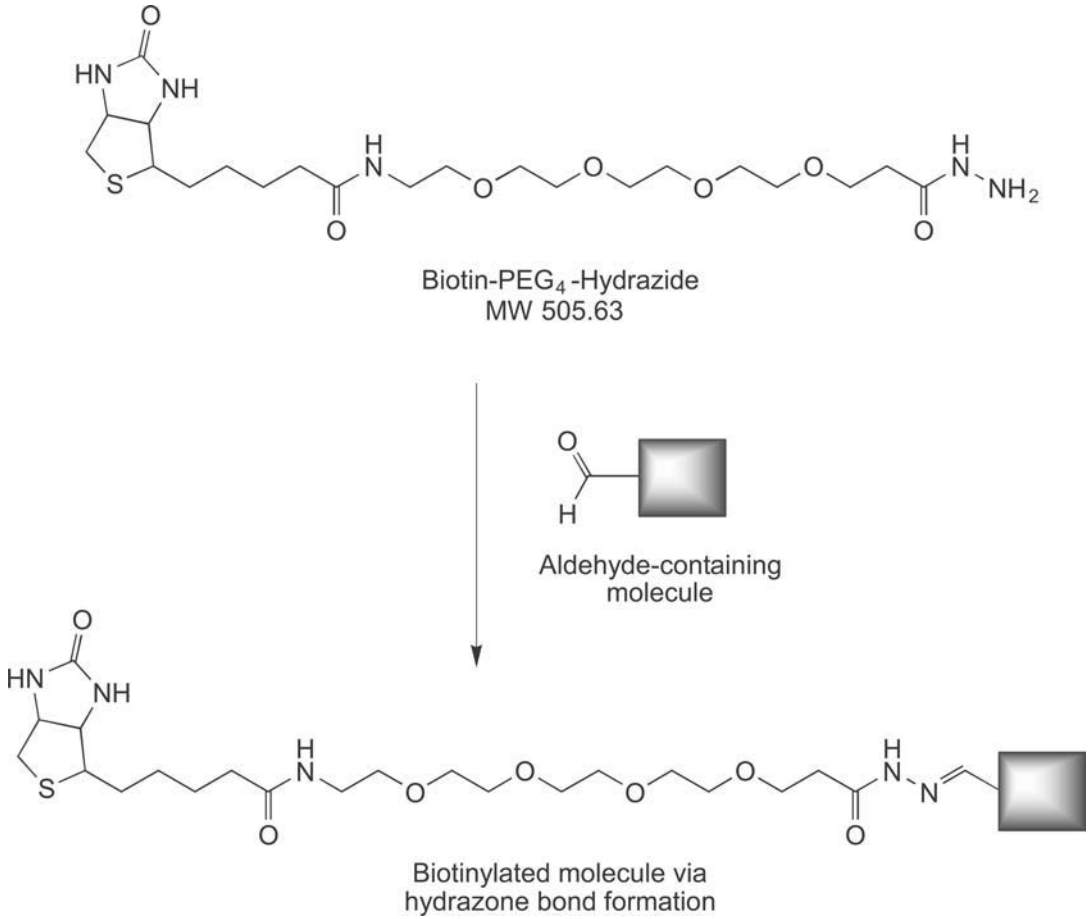


Figure 18.20 Biotin-PEG_4-hydrazide reacts with aldehyde-containing molecules to form a hydrazone linkage.

molecules using a carbodiimide reaction with EDC (Chapter 3, Section 1) or an active ester derivative (Chapter 2, Section 1). Like the other discrete PEG reagents, the hydrazide-PEG_4-biotin compound is very hydrophilic and won't promote aggregation or precipitation of labeled proteins. Aldehyde functionalities may be created on glycoproteins or other carbohydrates by oxidation using sodium periodate (Chapter 1, Section 2.2, 4.4–4.6) or by modification with SFB (Chapter 17, Section 2). The reducing end of sugars or glycans also may be labeled with these hydrazide reagents to produce a biotin–carbohydrate that is modified at only a single site.

Hydrazide-PEG_4-biotin can be used to label specifically glycoproteins on cell surfaces after mild periodate oxidation of the glycan structures (Wilchek and Bayer, 1987). The hydrophilic nature of the PEG spacer will prevent the biotinylation reagent from easily penetrating cell membranes, thus the labeling reaction is restricted to outer membrane glycoproteins. After biotinylation and cell lysis, the labeled proteins may be detected or isolated using (strept)avidin reagents (Jang and Hanash, 2003; Ding *et al.*, 2005; Handlogten *et al.*, 2005).

Hydrazide-PEG_4-biotin contains a 31.5 Å spacer consisting of four ethylene oxide units, which effectively imparts water solubility to the reagent. Other hydrazide biotinylation reagents that contain no spacer or a hydrocarbon spacer, such as biotin-hydrazide and biotin-LC-hydrazide, are water-insoluble and actually will lower the solubility of modified molecules. Hydrazide-PEG_4-biotin can be used to modify molecules without the tendency for aggregation or precipitation. In addition, the compound is stable in aqueous environments, as it contains no groups that are easily hydrolysable. A stock solution may be prepared in a water-miscible organic solvent such as DMAC, DMSO, or DMF to facilitate transfer of a small amount to an aqueous reaction.

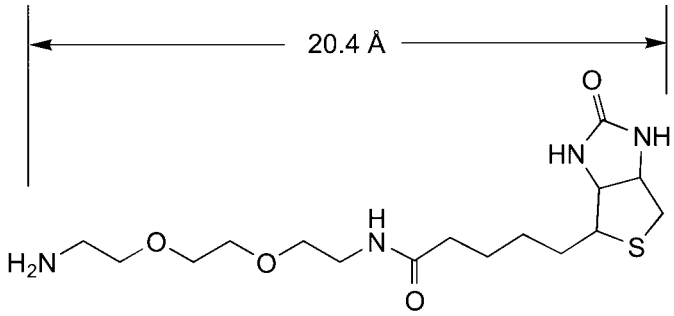
The following protocol describes a method for the periodate oxidation of a glycoprotein followed by biotinylation of the resultant aldehydes using hydrazide-PEG_4-biotin. Chapter 1, Section 4.6 describes an alternative protocol for the modification of glycans at their reducing ends with hydrazide compounds.

Protocol

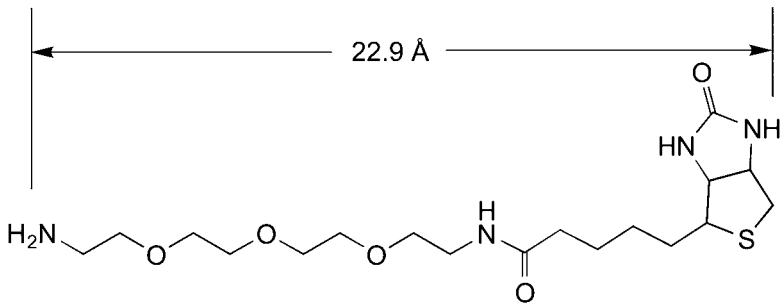
1. Dissolve a glycoprotein to be oxidized in 0.1 M sodium acetate, pH 5.5 (oxidation buffer), at a concentration of 2–10 mg/ml. PBS at physiological pH may be used for this reaction, as well. The use of cold buffers for the oxidation step will limit the extent of carbohydrate oxidation and the potential for protein oxidation.
2. Dissolve sodium *meta*-periodate in oxidation buffer at a concentration of 20 mM. Protect from light.
3. Add an equal volume of the glycoprotein solution to the periodate solution with mixing.
4. React for 10–20 minutes with gentle mixing and protected from light.
5. Quench the oxidation reaction by the addition of at least a 4-fold molar excess of *N*-acetylmethionine or sodium sulfite over the concentration of periodate in the reaction mixture (e.g., 40 mM). Pre-dissolve the quencher in buffer at a higher concentration prior to adding an aliquot of it to the reaction solution. React for 10 minutes. Alternatively, the oxidation reaction may be stopped by the removal of excess periodate by gel filtration using a desalting column.
6. Prepare a 50 mM solution of hydrazide-PEG_4-biotin in DMAC, DMSO, or DMF. Add a quantity of this solution to the purified, oxidized protein to provide at least a 10-fold molar excess of biotinylation reagent over the concentration of protein present.
7. React with mixing for 2 hours at room temperature.
8. The hydrazone bond can be reduced to stabilize the linkage by the addition of sodium cyanoborohydride to a final concentration of 50 mM. React for 30 minutes at room temperature with mixing. All operations with cyanoborohydride should be done in a fume hood. If the glycoprotein being modified is sensitive to disulfide reduction and potential denaturation, then this step should be avoided.
9. Purify the biotinylated glycoprotein by gel filtration or dialysis.

3.5. Biotin-PEG_n-Amine Compounds

Biotin compounds containing a PEG spacer that terminates in a primary amine can be used for the labeling of carboxylate molecules (Figure 18.21). Activated carboxylates, such as those



Biotin-PEG_2-Amine;
(+)-Biotinyl-3,6-dioxaoctanediamine
MW 374.50



Biotin-PEG_3-Amine;
(+)-Biotinyl-3,6,9-trioxaundecanediamine
MW 418.55

Figure 18.21 Biotin-PEG_n-amine compounds can be used to modify carboxylate- or aldehyde-containing compounds using a carbodiimide reaction.

containing an NHS ester, spontaneously react with the amines to give amide bond linkages. An active ester also may be formed *in situ* by the activation of carboxylates with EDC in the presence of NHS or sulfo-NHS (Chapter 3, Section 1) (Figure 18.22).

Biotin-PEG_2-amine contains a short, two-unit ethylene oxide cross-bridge that provides a 20.4 Å hydrophilic spacer, which has an amine on its end. Biotin-PEG_3-amine is identical except for one additional ethylene oxide unit. Both compounds are extremely water-soluble and can be used to label organic molecules or biomolecules containing carboxylates. Bronfman *et al.* (2003) used the biotin-PEG_n-amine compounds to label nerve growth factor (NGF) peptide on its carboxylates using EDC-mediated amide bond formation. The biotinylated growth factor then was used to study receptor internalization in live cells by probing with fluorescent streptavidin conjugates.

The following protocol can be used to biotinylate carboxylate-containing molecules in aqueous solution using the EDC/sulfo-NHS reaction.

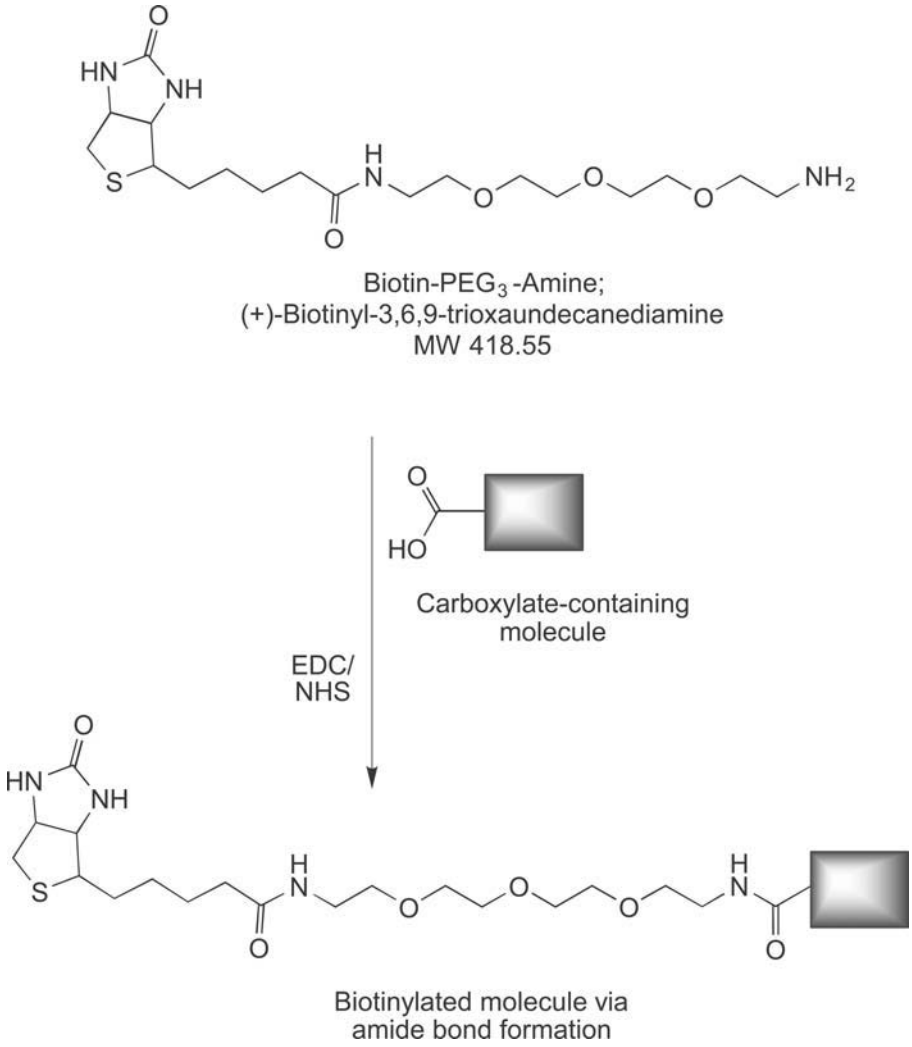


Figure 18.22 Biotin-PEG$_n$-amine can be used to add a biotin label to carboxylate-containing molecules using the EDC/(sulfo)NHS reaction, which forms a stable amide linkage.

Protocol

1. Dissolve a carboxylate-containing peptide or other molecule in 0.1 M MES, pH 5.0 (reaction buffer). Ideally, this molecule should contain only one carboxylate with no amines to direct biotinylation to a single site and prevent polymerization of it during the conjugation process. However, if a peptide is to be biotinylated that also has amine groups, then the use of a very high molar excess of the biotin-PEG$_n$-amine reagent during the reaction will limit the potential for peptide–peptide linking. The concentration of the carboxylate molecule should be low if it also has amines presence, but if it only has one or more carboxylates, then it can be prepared at higher concentration. For example, to use the biotin-PEG$_n$-amine compounds to biotinylate a protein, the concentration should be

on the order of 1–2 mg/ml so that a large excess of biotinylation agent can be added. For molecules that are sparingly soluble in aqueous solution, they may be dissolved first in ethanol and then added to the reaction buffer with mixing to make a final ethanol concentration of not more than 50 percent.
2. Dissolve the biotin-PEG$_n$-amine reagent in reaction buffer at a concentration of 25 mM.
3. Add a quantity of the biotin-PEG$_n$-amine solution to the solution containing the carboxylate molecule to achieve the desired molar excess. For molecules containing a single carboxylate to be modified, a 1.5- to 2-fold molar excess may be sufficient. However, for proteins or peptides that also contain competing amines, a much larger excess of biotin compound should be used (e.g., 100-fold excess). For instance, for protein biotinylation, add 120 µl of the biotin-PEG$_n$-amine solution per ml of the solution prepared in Step 1.
4. Immediately before use, dissolve EDC in reaction buffer at a concentration of 25 mM. Add 12 µl of this solution per ml of the combined solution from Step 2. Mix well.
5. React for 2 hours at room temperature or 4 hours at 4°C with gentle mixing.
6. Purify the biotinylated protein or molecule using dialysis or gel filtration. For small molecule biotinylation where these separation methods may not be appropriate, other procedures may have to be developed, such as reverse-phase chromatography or organic precipitation techniques.

3.6. Biotin–PEG$_3$–Benzophenone

Biotin–PEG$_3$–benzophenone is a biotinylation reagent with a hydrophilic spacer containing three ethylene oxide units and a photoreactive group at its end (Quanta BioDesign). The benzophenone is activated by UV light to an extremely reactive triplet-state ketone, which can insert into C—H, N—H, and other structures, resulting in a covalent bond (Chapter 2, Section 7.2). The reaction is one of the most efficient photoreactive conjugation mechanisms available (Campbell and Gioannini, 1979). Thus, this reagent provides a method of adding a biotin group to molecules that don't contain typical functionalities useful for bioconjugation. This may include polymeric surfaces or organic molecules lacking reactive targets (Figure 18.23).

The presence of the PEG$_3$ spacer in this compound provides water solubility to the biotin arm, whereas the benzophenone group should associate with more hydrophobic regions or surfaces, which may be ideal for the biotinylation photoreaction. The reagent can be used by dissolving it in an aqueous buffer suitable for use with whatever substance is to be biotinylated. After mixing this solution with the target molecule or surface, exposure to UV light will initiate the conjugation reaction. Unlike other photoreactive groups, a benzophenone doesn't undergo decomposition to an inactive form if it doesn't couple to target molecules. Instead, it degrades from the photo-excited state back to its initial state, so it can be once again photolyzed to an active state. This process increases the likelihood that the benzophenone will couple to a target molecule during the photoreaction. See Chapter 5, Section 4.3 for an illustration of the benzophenone coupling reaction.

4. Discrete PEG Modification Reagents

Large polymer PEG reagents having molecular weights >2,000 Da have been used for over 20 years as modification agents for biological molecules (Chapter 25). These compounds often are

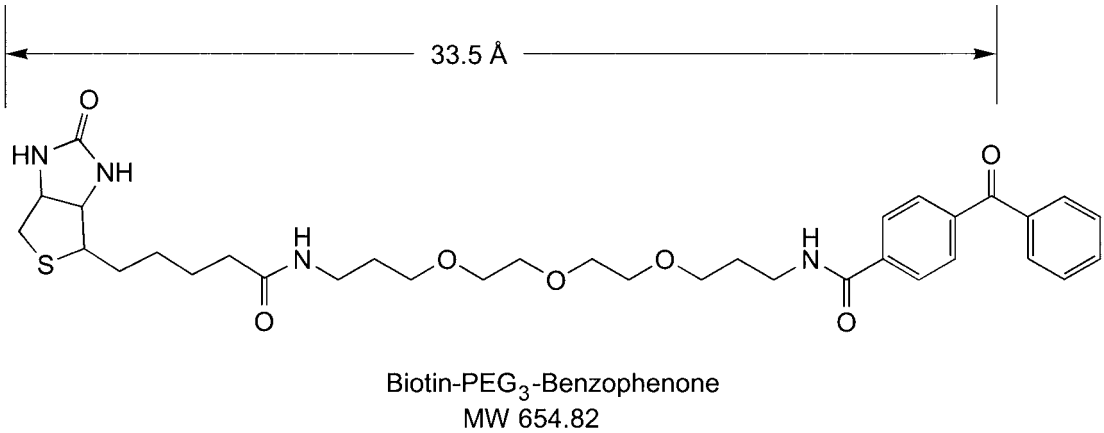


Figure 18.23 Biotin–PEG_3–benzophenone is a water-soluble photoreactive biotinylation reagent that can be used to add a biotin group to surfaces or molecules containing no easily derivatized functional groups.

used with a reactive group on one end and a blocked hydroxyl group on the other end (e.g., as the methyl ether). In addition, large, branched PEG molecules have been created to add more bulk or exclusion volume at a modification site, thus increasing the protective effect of the PEG molecule toward the biological molecule.

Discrete PEG compounds also have been developed in various reactive forms with methyl ether blocking groups on the terminal end (Thermo Fisher, Quanta BioDesign). Unlike the original PEG polymer reagents that display polydispersity, these PEG compounds are pure and consist of only one chain length per reagent type. The chain lengths in discrete PEG modifiers include, for example, polyethylene oxide repeat units of 3, 4, 8, 12, and 24. NHS–$mPEG_n$ modification reagents can be used directly to couple with amine-containing molecules or proteins through amide bond formation. This reaction occurs in aqueous buffers at physiological pH or slightly alkaline pH conditions. Conversely, maleimide–$mPEG_n$ reagents are designed to conjugate with thiol-containing molecules, and these may be used to target reduced disulfide bonds in proteins or thiols created on molecules using a thiolation reagent.

In addition, branched chain compounds have been developed consisting of a functional group or a reactive group followed by a PEG_4 chain, which then leads to three branches each having an $mPEG_{12}$ arm on them. Such compounds are expected to provide large exclusion volumes in aqueous solution to surround, protect, and solubilize modified molecules.

Another type of PEG modification reagent that has been developed contains a functional group on each end that can be used for conjugation purposes and which can be used to build structures on solid supports, surfaces, or on other molecules. Some of these reagents have been developed to contain an amine group on one end and a carboxylate on the other end. Such PEG-based amino acids can be used as spacer arms to mask surfaces or provide highly hydrophilic tethers for the attachment of affinity ligands. A thiol-PEG_n-carboxylate, for instance, can be used to modify metal particles or surfaces through dative binding of the thiol to the metal and then create PEG–carboxylic acids for further conjugation.

Figures 18.24 through 18.26 illustrate these PEG-based modification reagents. The methods for their use follow the same general protocol guidelines as discussed in previous sections

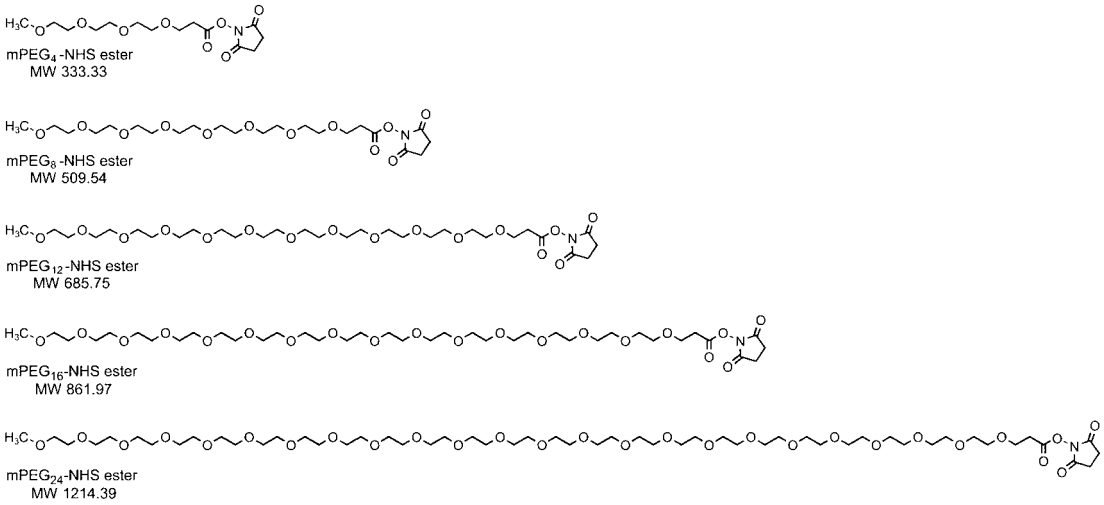


Figure 18.24 Discrete PEGylation reagents are available to provide a range of different chain lengths for adding mPEG modification arms to biomolecules. They also can be used to add water-soluble mPEG groups to organic molecules that are normally not very soluble in aqueous solution. The NHS ester end of the mPEG compounds reacts with amine-containing molecules to form amide bonds, leaving the mPEG chain to interact with the aqueous environment.

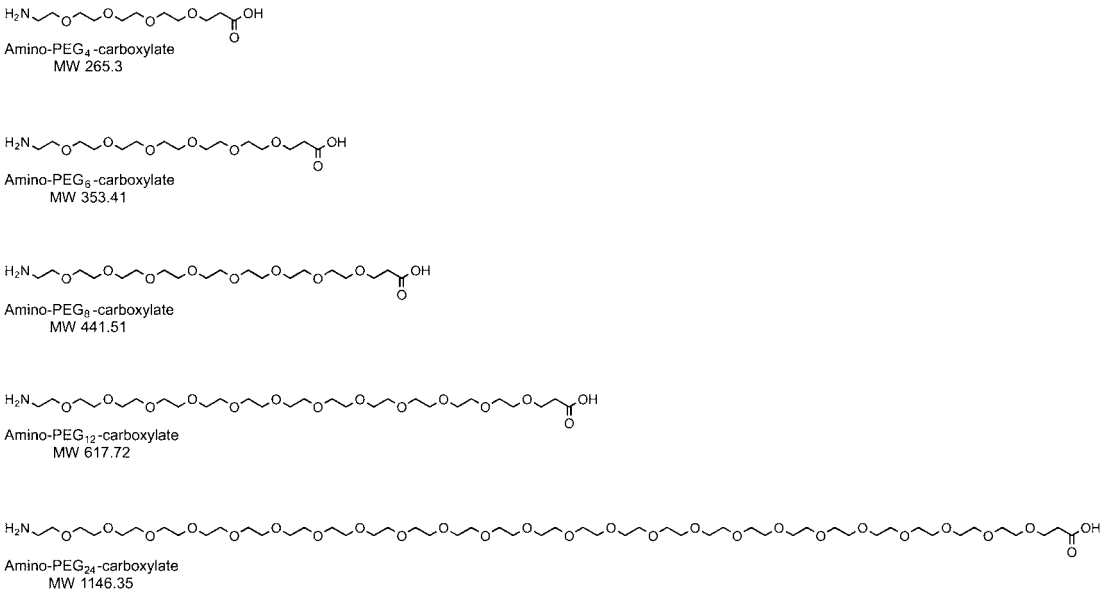


Figure 18.25 Amino-PEG$_n$-carboxylate compounds contain a primary amine on one end and a carboxylate group on the other end. They can be used to add water-soluble spacer arms to molecules or surfaces. Using an amine-reactive group, the amino-PEG$_n$-carboxylate compound can be coupled via an amide bond, thus leaving the carboxylate end free for further conjugation reactions. Avoid the use of single-step EDC conjugation reactions, as this will polymerize the amino-PEG$_n$-carboxylate by reacting with both ends.

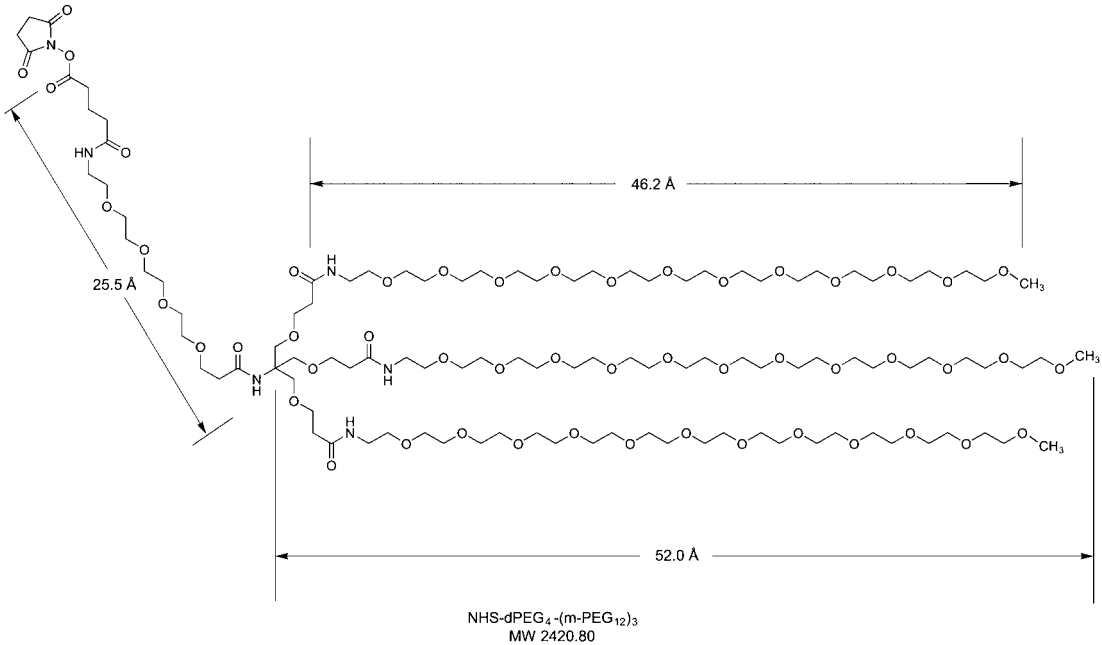


Figure 18.26 The branched PEGylation compound NHS-dPEG$_4$-(mPEG$_{12}$)$_3$ contains three mPEG arms, which provide an increased sphere of hydration around modified molecules compared to straight-chain PEGylation compounds.

of this chapter for the corresponding reactive group or functional group. When modifying biomolecules with mPEG-based reagents, a series of modification levels should be investigated to determine the optimal performance in an intended application. For surface or particle modification, reference should be made to Chapter 14, especially the section on covalent coupling to particles.

PART III

Bioconjugate Applications

The technology of bioconjugation has affected every conceivable discipline in the life sciences. The application of a myriad of available chemical reactions and reagent systems for creating novel complexes with unique activities has made possible the assay of minute quantities of substances, the *in vivo* targeting of molecules, and the modulation of specific biological processes. Modified or conjugated molecules also have been used for purification, detection, or location of specific substances, and in the treatment of disease.

Crosslinking and modifying agents can be applied to alter the native state and function of peptides and proteins, sugars and polysaccharides, nucleic acids and oligonucleotides, lipids, and almost any other molecule imaginable that can be chemically derivatized. Through careful modification or conjugation strategies, the structure and function of proteins can be investigated, active site conformation discovered, or receptor–ligand interactions revealed. Some of these techniques are so well characterized and standardized that general protocols can be used with broad application and with excellent prospects for success. The following sections describe how to prepare modified or conjugated biological macromolecules for use in specific applications. The chosen applications represent some of the most popular uses of these reagent systems, but are by no means exhaustive.

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19

Preparation of Hapten–Carrier Immunogen Conjugates

This chapter describes the design, preparation, and use of hapten–carrier conjugates used to elicit an immune response toward a coupled hapten. The chemical reactions discussed for these conjugations are useful for coupling peptides, proteins, carbohydrates, oligonucleotides, and other small organic molecules to various carrier macromolecules. The resultant conjugates are important in antibody production, immune response research, and in the creation of vaccines.

1. The Basis of Immunity

The essence of adaptive immunity is the ability of an organism to react to the presence of foreign substances and produce components (antibodies and cells) capable of specifically interacting with and protecting the host from their invasion. An "antigen" or "immunogen" is the name given for a substance which is both able to elicit this type of immune response and also is capable of interacting with the sensitized cells and antibodies which are manufactured against it.

The immune system has two basic components which respond to a challenge of a foreign substance: a cellular response mediated by T lymphocytes and a humoral response mediated by secreted proteins called antibodies produced by B-lymphocytes, also called plasma cells. The B-lymphocytes recognize antigens through cell-surface immunoglobulins that bind to discrete chemical and structural epitopes on the antigen molecule. Each B cell possesses surface immunoglobulin of a single type (i.e., is monoclonal) and has a binding capability that is directed against a discrete epitopic target.

Antigen binding by a complementary immunoglobulin molecule on the surface of B cells starts a process of cellular internalization of the foreign substance by pinocytosis. Once internalized by endosomes, systematic processing of the antigen takes place which breaks it down into smaller components.

At this point, the endosome may fuse with vesicles containing newly synthesized or recycling major histocompatibility complex (MHC) antigens. Some of the partially degraded antigenic fragments may form a complex with the MHC and be transported back to the cell surface. There they are "presented" to the circulating T helper (T_h) cells which contain receptors able to bind specifically to particular structural and chemical characteristics of the degraded antigen–MHC complex. If a T_h cell recognizes and binds to the presented antigen on the

surface of the antigen presenting cells (APC), the T_h cell proliferates and begins to produce various lymphokines. Finally, the recognition and binding of the presented antigen by the T_h cells, coupled with the release of lymphokines, stimulates the associated B cells to proliferate and produce antibodies which recognizes the intact antigen (Germain, 1986; Pier *et al.*, 2004).

Antigens usually are macromolecules that contain distinct antigenic sites or "epitopes", which can be recognized and interact with the various components of the immune system. They can exist as individual molecules composed of synthetic organic chemicals, proteins, lipoproteins, glycoproteins, RNA, DNA, polysaccharides—or they may be parts of cellular structures (bacteria or fungi) or viruses (Male *et al.*, 1987; Harlow and Lane, 1988).

Small molecules like short peptides, although normally able to interact with the products of an immune response, often cannot cause a response on their own. These "haptens", as they are called, actually are incomplete antigens, and while not able by themselves to cause immunogenicity or to elicit antibody production, they can be made immunogenic by coupling them to a suitable carrier molecule (Figure 19.1). Carriers typically are antigens of higher-molecular weight that are able to cause an immunological response when administered *in vivo*.

Antibodies typically are able to recognize peptide sequences as small as 5–6 amino acids in length. For instance, IgE auto-antibodies were found to have clinical significance in multiple sclerosis by binding specifically to short 5- and 6-amino acid epitopes on the surface of myelin proteins (Mikol *et al.*, 2006).

In an immune response, antibodies are produced and secreted by the B-lymphocytes in conjunction with the T_h cells. In the majority of hapten–carrier systems, the B cells end up producing antibodies that are specific for both the hapten and the carrier. In these cases, the T lymphocytes will have specific-binding domains on the carrier, but will not recognize the hapten alone. In a kind of synergism, the B- and T-cells cooperate to induce a hapten-specific antibody response. After such an immune response has taken place, if the host is subsequently challenged with only the hapten, usually it will respond by producing hapten-specific antibodies from memory cells formed after the initial immunization. For a review of immunobiology (see Janeway, 2004).

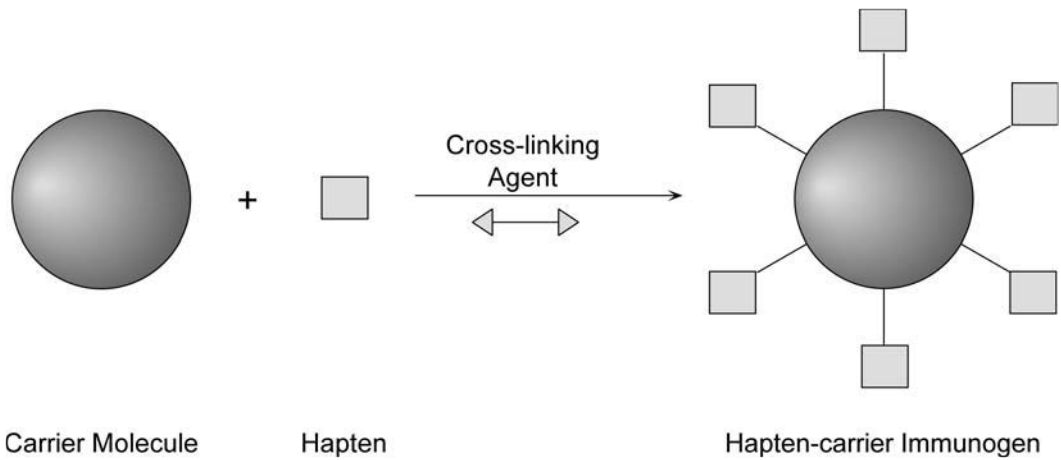

Figure 19.1 Immunogens are made by the crosslinking of a hapten molecule with a carrier using a conjugation reagent.

Synthetic haptens mimicking some critical epitopic structures on larger macromolecules are often conjugated to carriers to create an immune response to the larger 'parent' molecule. For instance, short peptide segments can be synthesized from the known sequence of a viral coat protein and coupled to a carrier to induce immunogenicity toward the native virus. This type of synthetic approach to immunogen production has become the basis of much of the current research into the creation of vaccines.

The complete picture of the immune system is much more complex than this brief discussion can justly describe. In many instances, merely creating a B cell response by using synthetic peptide-carrier conjugates, however well designed, will not always guarantee complete protective immunity toward an intact antigen. The immune response generated by a short peptide epitope from, say, a larger viral particle or bacterial cell may only be sufficient to generate memory at the B cell level. In these cases, it is generally now accepted that a cytotoxic T-cell response is a more important indicator of protective immunity. Designing peptide immunogens with the proper epitopic binding sites for both B-cell and T-cell recognition is one of the most challenging research areas in immunology today.

Hapten–carrier conjugates also are being used to produce highly specific monoclonal antibodies that can recognize discrete chemical epitopes on the coupled hapten. The resulting monoclonals often are used to investigate the epitopic structure and interactions between native proteins. In many cases, the haptens used to generate these monoclonals are again small peptide segments representing crucial antigenic sites on the surface of larger proteins. Monoclonals developed from known peptide sequences will interact in highly defined ways with the protein from which the sequence originated. These antibodies then can be used, for example, as competitors to the natural interactions between a receptor and its ligand. Thus, using antibodies generated from hapten–carrier conjugates, information can be obtained as to the precise sites of binding between macromolecules.

The preparation of hapten–carrier conjugates using peptide sequences can be controlled to produce immunogens that generate high-affinity antibodies when administered *in vivo*. Pedersen *et al*. (2006) determined that antibody titers increased in response to increasing the peptide-to-carrier ratio of conjugation. However, just the opposite effect was found for generating high affinity antibodies. The lower the peptide-to-carrier conjugation ratio, the higher the relative affinity of the antibodies produced. In addition, it also was found that coupling peptides to the carrier through a central amino acid residue caused higher antibody titers than using a terminal amino acid residue for conjugation. For this reason, for the preparation of particular immunogen conjugates, several ratios and methods of conjugation may have to be investigated to result in the optimal level and affinity of antibodies produced.

2. Types of Immunogen Carriers

The most commonly used carriers are all highly immunogenic, large molecules that are capable of imparting immunogenicity to covalently coupled haptens. Some of the more useful ones are proteins, but other carriers may be composed of lipid bilayers (liposomes), synthetic or natural polymers (dextran, agarose, poly-L-lysine), or synthetically designed organic molecules (i.e., dendrimers, see Chapter 7). The criteria for a successful carrier molecule are the potential for immunogenicity, the presence of suitable functional groups for conjugation with a hapten,

reasonable solubility properties even after derivatization—although this is not an absolute requirement, since precipitated molecules can be highly immunogenic—and lack of toxicity *in vivo*.

Some synthetic carriers actually are designed to have low immunogenicity on their own to minimize the potential for antibody production against them. When a hapten is coupled to these molecules, the immune response is directed principally toward the modification, not at the carrier. This design approach guides most of the immune response toward the desired target and minimizes the production of carrier-specific antibodies.

2.1. Protein Carriers

The first carrier molecules used for immunogen conjugation were proteins. A foreign protein administered *in vivo* by any one of a number of potential routes nearly assured the elicitation of an immune response. In addition, protein carriers could be chosen to be highly soluble and possessed of abundant functional groups that could facilitate easy conjugation with a hapten molecule. When proteins are used as carriers in immunogen complex, the conjugates can be injected in any animal except the animal of origin for the carrier protein itself. In other words, the use of bovine serum albumin (BSA) would not be suitable for administration into cows, since self-proteins would not be expected to elicit good immune responses, even when attached with hapten molecules.

The most common carrier proteins in use today are keyhole limpet hemocyanin (KLH; MW 4.5×10^5 to 1.3×10^7), BSA (MW 67,000), aminoethylated (or cationized) BSA (cBSA), thyroglobulin (MW 660,000), ovalbumin (OVA; MW 43,000), and various toxoid proteins, including tetanus toxoid and diphtheria toxoid. Other proteins occasionally used include myoglobin, rabbit serum albumin, immunoglobulin molecules (particularly IgG) from bovine or mouse sera, tuberculin purified protein derivative, and synthetic polypeptides such as poly-L-lysine and poly-L-glutamic acid.

KLH

Perhaps the most popular carrier protein is KLH. The hemocyanin from keyhole limpets (the mollusk *Megathura crenulata*) is the oxygen-carrying protein of these primitive sea creatures. KLH is an extremely large, multi-subunit protein that contains chelated copper of non-heme origin. In concentrated solutions above pH 7.0, it displays a characteristic opalescent blue color that betrays its near insolubility and copper prosthetic groups. In acidic solutions, the blue color changes to green. At physiological pH, the protein exists in various subunit aggregate states of large molecular weight. For instance, in Tris buffer at pH 7.4 it is known to associate in five different aggregate forms (Senozan *et al*., 1981). In highly alkaline or acidic environments, KLH disassociates into subunits (Hersckovits, 1988). The protein exhibits increased immunogenicity when it is disassociated into subunits, probably due to exposure of additional epitopic sites to the immune system (Bartel and Campbell, 1959). The intact protein usually creates considerable light-scattering or iridescent effects due to its size and almost colloidal nature in aqueous solutions. Subunits of KLH that are highly soluble in aqueous solution are available commercially (Thermo Fisher, Biosyn). KLH is a frequent choice for developing immunogen conjugates, especially for the treatment of cancer (Curigliano *et al*., 2006; Sabbatini and Odunsi, 2007).

Since keyhole limpets are marine creatures existing in a high-salt environment, native KLH maintains its best stability and solubility in buffers containing at least 0.9 M NaCl (not 0.9 percent). As the concentration of NaCl is decreased below about 0.6 M, the protein begins to precipitate and denature. Conjugation reactions using multi-subunit KLH, therefore, should be done under high-salt conditions to preserve the solubility of the hapten–carrier complex. KLH used in the form of discrete subunits does not have this requirement of high salt to maintain solubility.

Native, multi-subunit KLH also should not be frozen. Freeze-thaw effects cause extensive denaturation and result in considerable amounts of insoluble material. Commercial preparations of native KLH are typically freeze-dried solids that no longer fully dissolve in aqueous buffers and do not display the protein's typical blue color due to loss of chelated copper. The partial denatured state of these products often makes conjugation reactions difficult.

KLH contains an abundance of functional groups available for conjugation with hapten molecules. On a per-mole basis (using an average multi-subunit MW of 5,000,000 D), KLH has over 2,000 amines from lysine residues, over 700 sulfhydryls from cysteine groups, and over 1,900 tyrosines. Activation of the protein with succinimidyl-4-(*N*-maleimidomethyl)cyclo hexane-1-carboxylate (SMCC) (Section 5, this chapter) typically results in 300–600 maleimide groups per molecule for coupling to sulfhydryl-containing haptens.

The preparation of immunogen conjugates often requires the coupling of a sparingly soluble hapten to a carrier molecule. Pre-dissolving the hapten in an organic solvent and adding an aliquot of this solution to an aqueous reaction mixture typically is done to maintain at least some solubility of the hapten in the conjugation solution. Dimethyl sulfoxide (DMSO) may be used for this purpose with KLH while maintaining very good solubility characteristics of the protein as well as the hapten. KLH is completely soluble in 50 percent (v/v) DMSO, becomes cloudy at a level of 60 percent, and definitely precipitates at 67 percent. Therefore, conjugation reactions may be done by adding a volume of aqueous KLH to an equal volume of hapten dissolved in DMSO. Care should be taken, however, to avoid buffer salt precipitation upon addition of organic solvent.

BSA and cBSA

BSA (MW 67,000) and cationized BSA (cBSA) are highly soluble proteins containing numerous functional groups suitable for conjugation. Even after extensive modification with hapten molecules these carriers usually retain their solubility. The exception to this statement is when hydrophobic peptides or other sparingly soluble molecules are conjugated to the proteins. Modification of any carrier with hydrophobic haptens may cause enough masking of the hydrophilic surface to result in precipitation. Depending on the degree of precipitation, such conjugates often are useful in generating an immune response. To limit the production of insoluble complexes, however, the conjugation reaction can be scaled back to reduce the level of carrier modification.

BSA possesses a total of 59 lysine ε-amine groups (with only 30–35 of these typically available for derivatization), 1 free cysteine sulfhydryl (with an additional 17 disulfides buried within its three-dimensional structure), 19 tyrosine phenolate residues, and 17 histidine imidazole groups. The presence of numerous carboxylate groups gives BSA its net negative charge (pI 5.1).

cBSA is prepared by modification of its carboxylate groups with ethylene diamine (Chapter 1, Section 4.3) (Figure 19.2). Controlled aminoethylation using the water-soluble carbodiimide EDC results in blocking many of BSA's aspartic and glutamic acid side chains (and possibly the

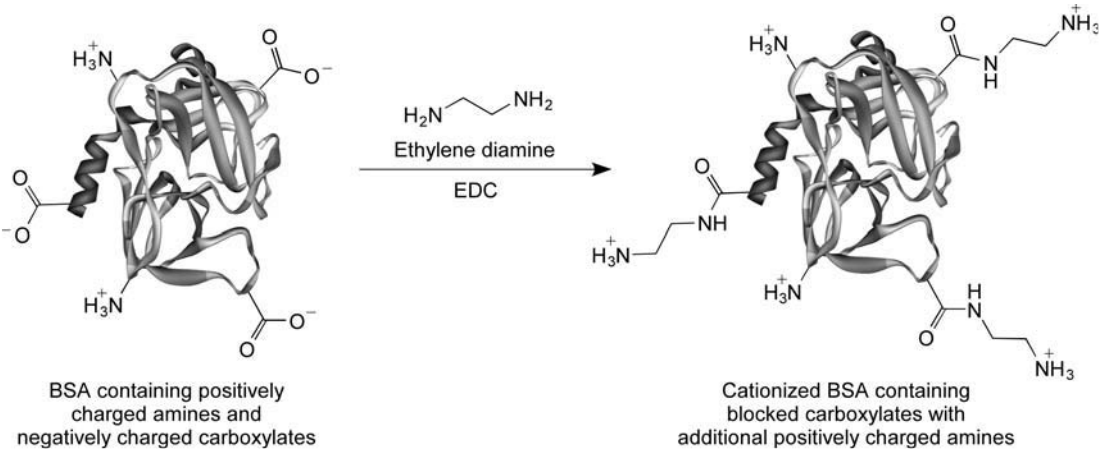

Figure 19.2 cBSA is formed by the reaction of ethylene diamine with BSA using the water-soluble carbodiimide EDC. Blocking of the carboxylate groups on the protein combined with the addition of terminal primary amines raises the pI of the molecule to highly basic values.

C-terminal carboxylate), forming an amide bond with a 2-carbon spacer containing a terminal primary amine group. Since the negative charge contributions of the native carboxylates are masked and positively charged amines are created in their place, the result of this process is a significant rise in the protein's pl. Cationization performed according to published procedures alters the net charge of BSA from a pl of about 5.1 (Cohn *et al*., 1947) to over pl 11.0 (Muckerheide *et al*., 1987).

The highly positive charge of cBSA dramatically increases its immunogenicity. The positive character of the molecule aids in its binding to APC *in vivo*, the first step in antibody production. The protein thus gets incorporated into the APCs faster than molecules having lower pI values. It also gets processed at an accelerated rate, producing a quicker immune response, and one that occurs with greater concentrations of specific antibody (Domen *et al*., 1987; Muckerheide *et al*., 1987b; Apple *et al*., 1988; Domen and Hermanson, 1992; Chen *et al*., 2002).

cBSA used as a carrier protein also induces a similar increase in the production of antibody against any attached hapten molecules. Even when haptens are coupled through cBSA's amine residues, the overall charge of the molecule remains basic enough to augment the immune response beyond that usually obtained using other carriers. This augmentation occurs even when the attached molecule is not merely a hapten, but a larger antigen macromolecule. Conjugation of a complete antigen (a molecule able to generate an immune response on its own) to cBSA causes an increased immune response against the antigen beyond that normally obtainable for the native antigen administered in unconjugated form (Domen and Hermanson, 1992).

The effectiveness of cBSA as a carrier for peptides was investigated using arginine vasopressin (AV) as the hapten. Figure 19.3 shows the antibody concentration resulting after injection of the AV–cBSA conjugate intraperitoneally (i.p.) into BDF_1 female mice. As a control, native BSA (nBSA) was similarly conjugated with AV and administered in a second set of mice under identical conditions. The antibody concentrations in the sera were monitored periodically by enzyme-linked-immunosorbent assay (ELISA). The antibody response resulting from a set of mice injected with unconjugated peptide was subtracted in all cases. All injections were done

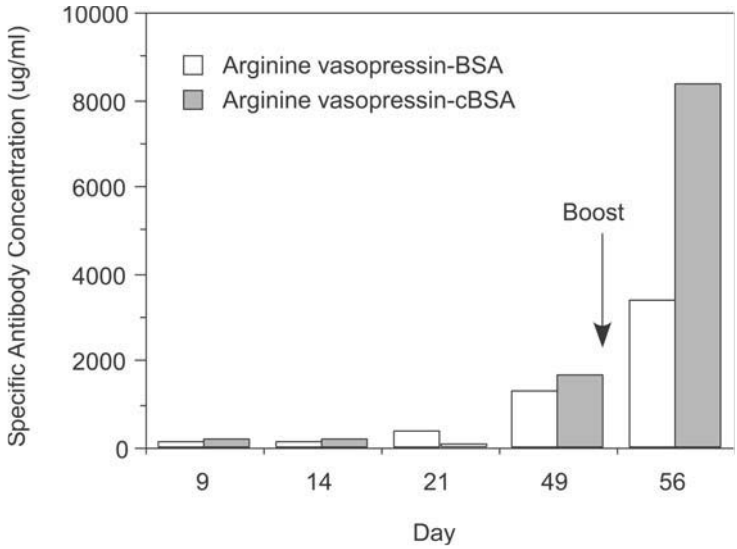

Figure 19.3 The effectiveness of cBSA as an immunogen can be seen by the comparison of specific antibody response in mice to AV coupled to both nBSA cBSA. The quantity injected was standardized according to the amount of AV present. The cationized carrier results in higher concentrations of antibody produced against the peptide than the immunogen made with nBSA.

using 100 μg of conjugate mixed with an equal volume of alum (22.5 mg/ml aluminum hydroxide) as adjuvant.

After the boost, the group of mice receiving the AV–cBSA conjugate generated over twice the antibody response as the group receiving the peptide conjugated to nBSA.

In a similar study, OVA conjugated to cBSA was compared to the same protein conjugated to nBSA and also OVA administered in an unconjugated form in mice. Figure 19.4 shows that before and after the boost, the OVA–cBSA conjugate resulted in much higher antibody concentrations than either the OVA–nBSA conjugate or OVA injected in an unconjugated form. Similar results were obtained for a conjugate of human IgG with cBSA (Figure 19.5).

A corollary to the use of cBSA as a carrier protein is that its increased immune response often abrogates the use of complete Freund's adjuvant, which is a source of concern because of its potential side-effects in animals. A relatively innocuous mixture with alum is usually all that is required as adjuvant to result in good antibody production.

As mentioned previously for KLH, DMSO may be used to solubilize hapten molecules that are rather insoluble in aqueous environments. Conjugation reactions may be done in solvent/aqueous phase mixtures to maintain some solubility of the hapten once it is added to a buffered solution. BSA remains soluble in the presence of up to 35 percent DMSO, becomes slightly cloudy at 40 percent, and precipitates at 45 percent (v/v).

Thyroglobulin and OVA

Thyroglobulin and ovalbumin (OVA) are used less often as carriers, but they are particularly valuable as non-relevant carriers in ELISA tests designed to measure the antibody response

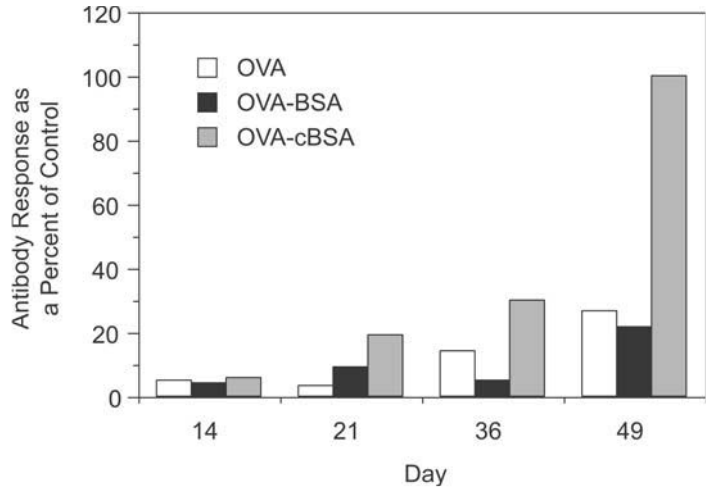

Figure 19.4 cBSA even can increase the specific antibody response to large proteins coupled to it. This graph shows a comparison of the relative antibody response in mice to injections of OVA, either in an unconjugated form or conjugated to nBSA or cBAS. The quantity injected was standardized according to the amount of OVA present. The highly basic cBSA molecule modulates the immune response to enhance the production of antibodies toward even proteins conjugated with it.

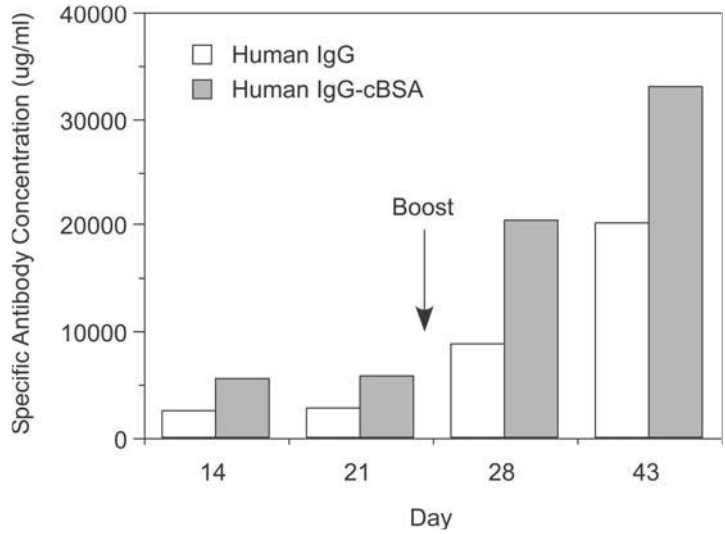

Figure 19.5 Human IgG was injected in mice either in an unconjugated form or crosslinked with cBSA. The quantity injected was standardized according to the amount of IgG present. A greater antibody response was obtained using the cBSA conjugate.

after injection of an immunogen conjugate. Since an antibody response would be directed both against the carrier and the attached hapten, an ELISA done to quantify specific antibody that only interacts with the hapten must not utilize the same carrier in the conjugate coated on the microplates. If the identical carrier conjugate is used for the ELISA as was used in the

original immunization, the test results will be skewed by the contribution of carrier-specific antibodies. For this reason, a non-relevant carrier—one that is not recognized by the products of the immune response—must coupled with hapten and used for the ELISA test.

Since OVA and BSA possess some immunologically similar epitopes, a population of the antibodies produced against one often will cross-react against the other. Therefore, OVA cannot function as a non-relevant carrier for BSA and vice versa. Either OVA or BSA, however, may be used as non-relevant carriers for KLH, thyroglobulin, or the various toxoid proteins used as immunogen conjugates.

OVA comprises about 75 percent of the total protein in hen egg whites. It is a phosphoprotein containing one *N*-glycosylation site and 386 amino acids. The protein contains 20 lysine residues, 14 aspartic acids, and 33 glutamic acid groups. This gives a total of 20 ε-amines, 1 N-terminal amine, 47 side-chain carboxylates, and 1 C-terminal carboxylate for conjugation reactions. The majority of acidic groups gives the protein a pI of 4.63. Additional sites of modification include 4 sulfhydryl groups, 10 tyrosines, and 7 histidine residues. OVA is sensitive to temperature (above 56°C), electric fields, and vigorous mixing. Care should be taken in handling the protein to prevent denaturation and subsequent precipitation.

One advantage of OVA is its extreme solubility characteristics in the presence of DMSO. A sparingly soluble hapten molecule may be dissolved in this solvent and added to an aqueous OVA reaction mixture to maintain solubility of the molecule during conjugation. OVA is soluble at up to 70 percent DMSO, becomes cloudy at 75 percent, and precipitates at 80 percent (v/v).

Thyroglobulin is a prohormone protein which is synthesized and stored in the thyroid gland. Specific proteolytic action on the protein *in vivo* causes the release of triiodothyronine and thyroxine, low-molecular weight amino acid derivatives that affect metabolic rate and oxygen consumption. Thyroglobulin is a large, multi-subunit protein composed of several polypeptide chains (MW 670,000). Its acidic pl (4.7) reflects the abundance of carboxylate groups. Thyroglobulin is also glycosylated, containing about 8–10 percent carbohydrate. Its use as an immunogen carrier protein is less frequent than that of KLH or BSA.

Tetanus and Diphtheria Toxoids

Toxoid proteins are biologically inactivated forms of native toxins. The most often used toxoid is tetanus toxoid, but diphtheria-derived toxoids and other proteins also are used occasionally (Anderson *et al*., 1989). Tetanus toxoid (MW 150,000) has 106 amine groups, 10 sulfhydryls, 81 tyrosine residues, and 14 histidines that may participate in conjugation reactions with hapten molecules (Bizzini *et al*., 1970). Diphtheria toxoid is derived from a protein secreted by certain strains of *Corynebacterium diphtheriae*. Its molecular weight is approximately 63,000 D (Collier and Kandel, 1971). Both protein toxoids can be used to couple haptens through any of the chemical reactions described in this section. They generate strong immunological responses *in vivo*.

2.2. Liposome Carriers

Liposomes are artificial structures composed of phospholipid bilayers exhibiting amphiphilic properties (Chapter 22). In complex liposome morphologies, concentric spheres or sheets of lipid bilayers are usually separated by aqueous regions that are sequestered or compartmentalized

from the surrounding solution. The phospholipid constituents of liposomes consist of hydrophobic lipid tails connected to a head constructed of various glycerylphosphate derivatives. The hydrophobic interaction between the fatty acid tails is the primary driving force for creating liposomal bilayers in aqueous solutions.

The morphology of a liposome may be classified according to the compartmentalization of aqueous regions between bilayer sheets. If the aqueous regions are sequestered by only one bilayer each, the liposomes are called unilamellar vesicles (ULV). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV forms are further classified as to their relative size, although rather crudely. Thus, there can be small unilamellar vesicles (SUV; usually less than 100 nm in diameter) and large unilamellar vesicles (LUV; usually greater than 100 nm in diameter). With regard to MLV, however, the bilayer structures cannot be easily classified due to the almost infinite number of ways each bilayer sheet can be associated and interconnected with the next one. MLVs typically form large complex honeycomb structures that are difficult to classify or reproduce.

The overall composition of a liposome—its morphology, composition (including a variety of potential phospholipids and the degree of its cholesterol content), charge, and any attached functional groups—can affect the antigenicity of the vesicle *in vivo* (Allison and Gregoriadis, 1974; Alving, 1987; Therien and Shahum, 1989). When liposomes are used as carriers for immunization purposes, the haptens or antigens usually are attached covalently to the head groups using various phospholipid derivatives and crosslinking strategies (Derksen and Scherphof, 1985). Most often, these derivatization reactions are done off of phosphatidylethanolamine constituents within the liposomal mixture. The primary amine modification of the glycerylphosphate head group of phosphatidylethanolamine provides an ideal functional group for activation and subsequent coupling of hapten molecules (Shek and Heath, 1983). Stock preparations of activated liposomes may be prepared and lyophilized to be used as needed in coupling hapten molecules (Friede *et al*., 1993). Conjugates of liposomes with peptides or other molecules have been used to target cells *in vivo* for disease therapy (Du *et al*., 2007). All of the amine-reactive conjugation methods discussed in this section may be used with phosphatidylethanolamine-containing liposomes; however see Chapter 22 for a more complete discussion of the unique considerations associated with conjugation of molecules to liposomes.

2.3. Synthetic Carriers

Synthetic molecules may be used as immunogen carriers if they are designed with the appropriate functional groups to couple hapten molecules. These carriers may consist of simple polymers such as poly-L-lysine, poly-L-glutamic acid, Ficoll, dextran, polyethylene glycol, or dendrimers (Lee *et al*., 1980; Fok *et al*., 1982; Boyle *et al*., 1983; Hopp, 1984; Wheat *et al*., 1985). Coupling of hapten molecules to the principle functional groups of these polymers can produce immunogenic conjugates that may be injected in animals to generate a specific antibody response.

Poly-L-lysine may be coupled to carboxylate-containing molecules using the carbodiimide conjugation procedure to yield amide linkages (Chapter 3, Section 1). Homobifunctional or heterobifunctional crosslinking agents also may be used with poly-L-lysine, such as in the use of sulfo-SMCC (Chapter 5, Section 1.3). The polymer can be used as well for coupling hapten molecules and subsequent coating of microplates for ELISA procedures (Gegg and Etzler, 1993). Conversely, poly-L-glutamic acid may be coupled to amine-containing haptens by the

same carbodiimide protocol. Ficoll and dextran carriers may be activated by mild sodium periodate oxidation to generate reactive aldehyde groups (Chapter 1, Section 4.4 and Chapter 25, Section 2.1). Coupling to amine-containing haptens then may be done by reductive amination (Chapter 2, Section 5). Polyethylene glycol chemistry involves alternate activation and coupling schemes that are addressed in Chapter 25, Section 1.

A unique synthetic molecule that can be used as a carrier is the so-called multiple antigenic peptide (MAP) (Posnett *et al*., 1988; Tam, 1988). The MAP core structure is composed of a scaffolding of sequential levels of poly-L-lysine. The dendritic molecule is constructed from a divalent lysine compound to which two additional levels of lysine are attached. The final MAP compound consists of a symmetrical, octavalent primary amine surface to which hapten molecules may be attached. Coupling of up to eight peptide haptens to the MAP core yields a highly immunogenic complex having a molecular weight of typically greater than 10,000. The nature of the MAP carrier makes it ideal for remarkably defined conjugates useful in vaccine development.

One particularly novel carrier was reported to consist of 50–70 nm colloidal gold particles of the type often used in cytochemical labeling techniques for microscopy (Pow and Crook, 1993) (Chapter 24). Adsorption of peptide antigens onto gold and subsequent injection of the complex into rabbits in an adjuvant mixture resulted in rapid production of antibody of extremely high titer. The resultant antibodies could be used in immunocytochemistry at dilutions from 1-in-250,000 down to 1-in-1,000,000, which is orders-of-magnitude beyond the dilutions typically used with lower-titer antibodies.

3. Carbodiimide-Mediated Hapten–Carrier Conjugation

The coupling chemistry used to prepare an immunogen from a hapten and carrier protein is an important consideration for the successful production and correct specificity of the resultant antibodies. The choice of crosslinking methodology is governed by the functional groups present on the carrier and the hapten as well as the orientation of the hapten desired for appropriate presentation to the immune system. An associated concern is the potential for antibody recognition and cross-reactivity toward the crosslinking reagent used to effect the conjugation. If antibodies are generated against the crosslinker bridge connecting the carrier with the hapten, then this may dilute the desired antibody response against the hapten. The use of a zero-length crosslinking procedure mediated by the water-soluble carbodiimide EDC eliminates this problem, since no bridging molecule is introduced between the hapten and carrier.

The reactions involved in an EDC-mediated conjugation are discussed in Chapter 3, Section 1.1 (*Note*: EDC is 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; MW 191.7 and is sometimes referred to as EDAC). The carbodiimide first reacts with available carboxylic groups on either the carrier or hapten to form a highly reactive *o*-acylisourea intermediate. The activated carboxylic group then can react with a primary amine to form an amide bond, with release of the EDC mediator as a soluble isourea derivative. The reaction is quite efficient with no more than 2 hours required for it to go to completion and form a conjugated immunogen.

Since most peptide haptens contain either amines or carboxylic groups available for coupling, EDC-mediated immunogen formation may be the simplest method for the majority of hapten–carrier protein conjugations. Figure 19.6 shows the coupling of a carrier protein to a short peptide molecule through its amine terminus. It should be kept in mind, however, that this type of conjugation may occur at either the C- or N-terminal of the peptide or at any carboxyl- or

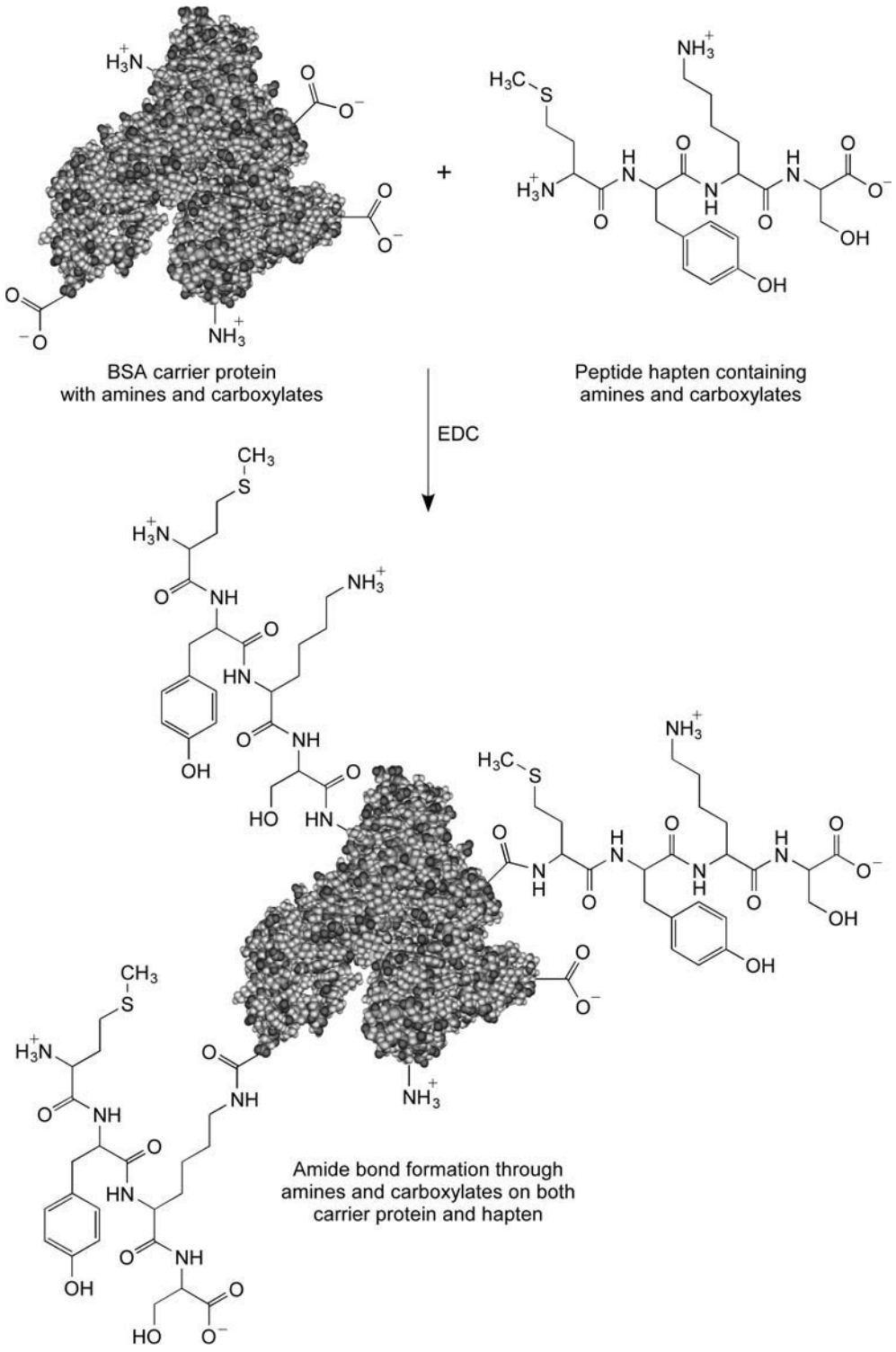

Figure 19.6 Peptide haptens are easily conjugated to carrier proteins using the water-soluble carbodiimide EDC.

amine-containing side chains. Therefore, this method probably should be avoided if a particularly interesting part of the peptide contains groups which may be blocked or undergo coupling using the carbodiimide reaction. Also, when using peptides rich in Lys, Glu, or Asp, an unacceptable level of hapten crosslinking may occur upon conjugation, and thus change the antigenic structure of the resulting immunogen. However, some crosslinking or polymerization of the peptide on the surface of the carrier actually may be beneficial to the immunogenicity of the peptide, and thus create an even greater antibody response. Some investigators even advocate using no carrier protein when a peptide hapten is involved: merely polymerizing the peptide in the presence of EDC may result in a complex of high enough molecular weight to be immunogenic by itself. In general, EDC coupling is a very efficient, one-step method for forming a wide variety of peptide-carrier protein immunogens.

Figure 19.7 shows the results of an EDC conjugation study comparing a reaction done at pH 4.7 (A) to one done at pH 7.3 (B and C), with and without added sulfo-NHS (see Chapter 3, Section 1.2). The graphs show the elution profiles of a gel filtration separation after conjugation. In each case, a blank run done without the addition of EDC illustrates the separation of the protein carrier (the first peak) from the lower-molecular weight peptide and reagent peak (the second peak). Decrease in the peptide peak is indicative of successful conjugation. Complete recovery of the total absorbance at 280 nm usually does not occur, presumably due to a decrease in the peptide's absorptivity as it is conjugated or polymerized. Staros' method of adding sulfo-NHS to form an intermediate active ester that subsequently reacts with an amine to form the amide bond does not work as well due to excessive conjugation (causing precipitation in most cases) and interference from the eluting sulfo-NHS peak. The reaction proceeds with similar yields at either acid or neutral pH. Thus, the efficiency of an EDC conjugation reaction is approximately the same from pH 4.7 to physiological conditions.

Figure 19.8 shows the result of the conjugation of the dipeptide tyrosyl-lysine to BSA using various concentrations of EDC. Again, the elution profile shows the gel filtration pattern resulting after the reaction. The first peak is the protein carrier while the second is the peptide. Progressive decrease in the peptide peak with increasing amounts of EDC added to the reaction mixture correlates to increased conjugation yields. A side reaction to EDC conjugation of haptens that contain both an amine and a carboxylate group is hapten polymerization. This is revealed in the movement of the peptide peak toward higher-molecular weights (e.g., decreased time of elution) with increasing amounts of EDC added to the reaction.

Figure 19.9 illustrates the conjugation of [Met5]-enkephalin with BSA using EDC. The gel filtration profile after crosslinking reveals that the peptide peak effectively disappears upon complete conjugation with the carrier protein. With nicely soluble peptides such as this one, the immunogen remains freely soluble even at high modification levels. For less soluble peptides or haptens, reducing the amount of EDC addition may be necessary to maintain solubility in the conjugate.

To illustrate the similarity of an EDC conjugation reaction using a different carrier protein, but the same peptide, Figure 19.10 shows the gel filtration separation after conjugation of [met5]-enkephalin to OVA. The uptake of peptide upon addition of EDC is almost identical to that observed when conjugating to BSA. This is logical, since on a per mass basis, there is very little difference between these proteins in the amount of amines or carboxylates available for conjugation.

Figure 19.11 shows the conjugation of tyrosyl-lysine to KLH using various concentrations of EDC. The elution profile shows the gel filtration pattern resulting after the reaction. Progressive decrease in the peptide peak (peak 2) with increasing amounts of EDC correlates to

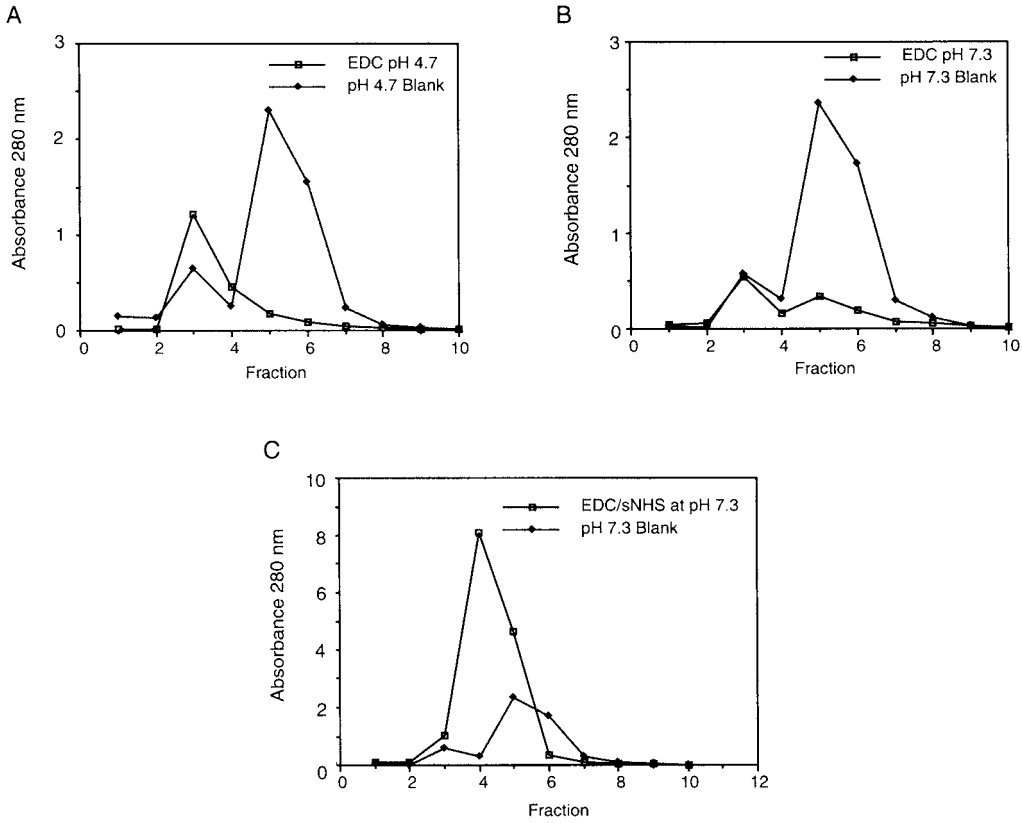

Figure 19.7 To assess the effectiveness of an EDC conjugation reaction of a peptide with a carrier protein, glycyl-tyrosine was coupled to BSA using various conditions. The graphs show a gel filtration profile on Sephadex G-25 after completion of the conjugation reaction. The first peak eluting off the column is the higher-molecular weight carrier, while the second peak is excess peptide. The elution profiles demonstrate that the carbodiimide reaction proceeds with nearly equal efficiency at pH 4.7 (A) or pH 7.3 (B). In each graph, a comparison is shown between the separation of peptide and carrier without addition of EDC and the same mixture after reaction with EDC. Depletion of the peptide peak in the EDC-containing elution profiles indicates uptake of glycyl-tyrosine in the carrier conjugate. Some polymerization of peptide is also possible using this method, as evidence by movement of the peptide peak toward higher-molecular weight elution points. Addition of sulfo-NHS to the reaction caused precipitation problems as well as obscuring the separation due to the absorbance of excess sulfo-NHS (C).

increased conjugation (or polymerization) yields. Despite the extremely high-molecular weight of KLH compared to the other commonly used carriers, the conjugation reaction using EDC again proceeds with virtually identical results to the similar study shown in Figure 19.8 using BSA as the carrier. In fact, superimposing the two studies on the same graph demonstrates the reproducibility of an EDC-facilitated reaction (Figure 19.12).

Due to the high-molecular weight of KLH and its solubility characteristics, the conjugation of this protein to some haptens can result in precipitation of the complex. This is especially true if the level of EDC addition is similar to the EDC concentrations used with lower-molecular weight carriers such as BSA or OVA. Figure 19.13 shows the elution profile

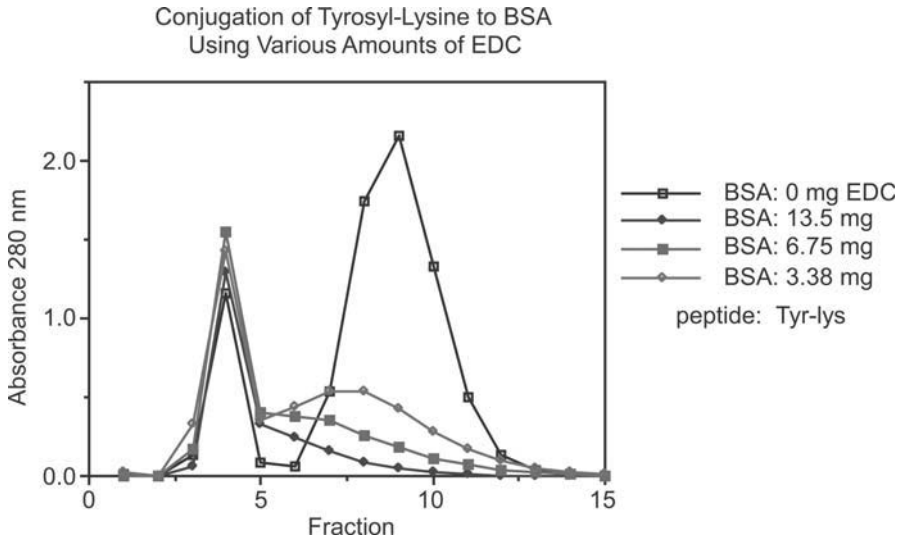

Figure 19.8 To study the conjugation of peptides to carriers using different levels of EDC, tyrosyl-lysine was conjugated to BSA and separated after the reaction by chromatography on a Sephadex G-25 column. As the EDC level was increased in the reaction, more peptide reacted and the peptide peak (the second peak) was depleted. The absorbance of the carrier peak (the first one) increases as more peptide is conjugated.

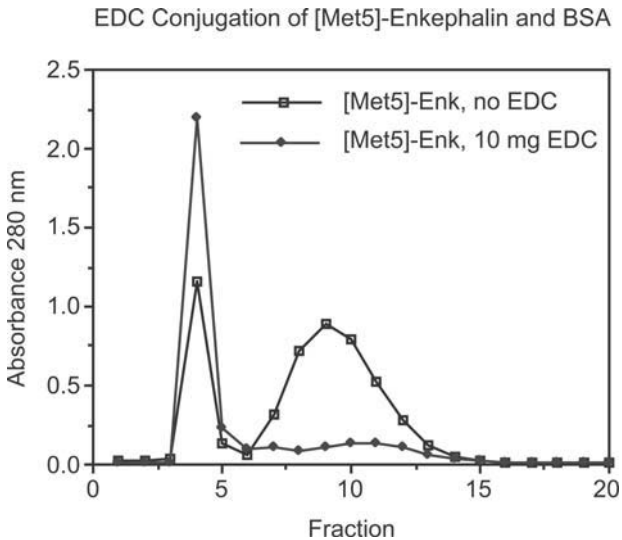

Figure 19.9 Conjugation of the biological peptide [Met5]-enkephalin to BSA using EDC. The graph shows the gel filtration profile (on Sephadex G-25) after completion of the conjugation reaction. A blank run with no added EDC was done to illustrate the peak absorbance that would be obtained if no conjugation took place. With addition of 10 mg of EDC to a reaction mixture consisting of 2 mg of BSA plus 2 mg of peptide, nearly complete conjugate formation was obtained.

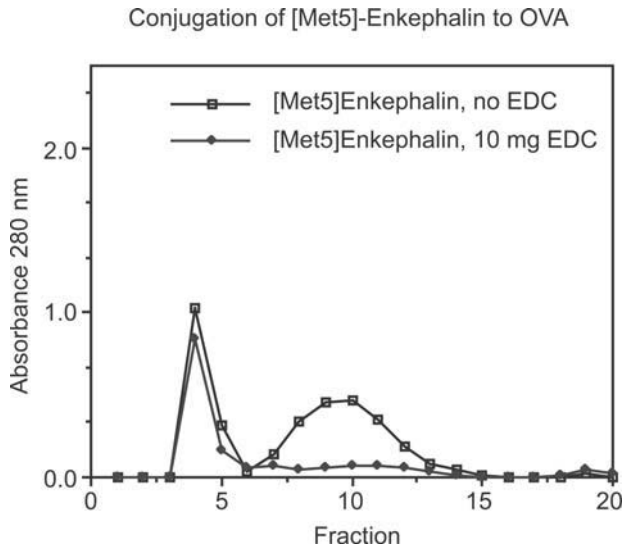

Figure 19.10 To illustrate the consistency of an EDC-mediated reaction, [Met5]-enkephalin was conjugated to OVA using conditions identical to those described for BSA in Figure 19.9. Note the similarity in the degree of conjugate formation.

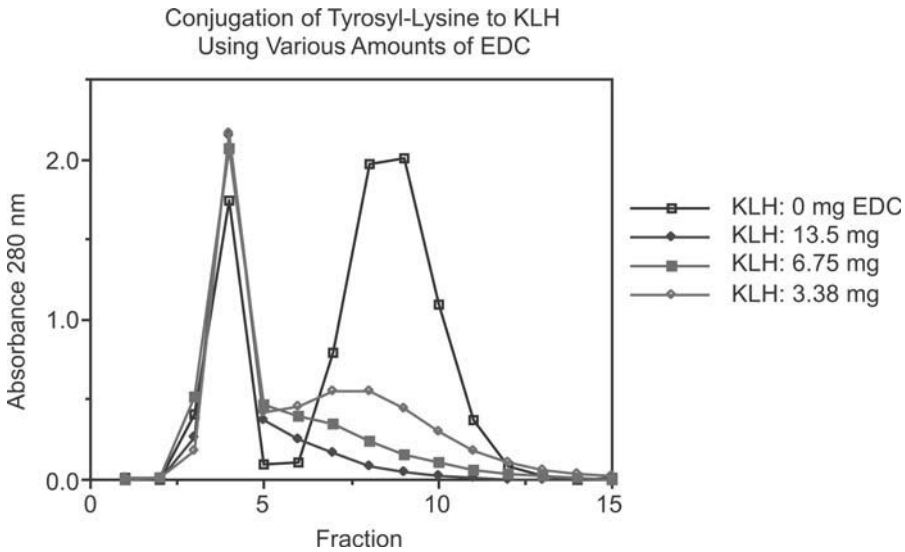

Figure 19.11 The EDC conjugation of tyrosyl-lysine to KLH is illustrated by the gel filtration pattern on Sephadex G-25 after the reaction. The first peak is the carrier protein and the second peak is the peptide. A blank containing no EDC is also shown to provide baseline peak heights that would be obtained if no crosslinking occurred. When more EDC was added, more peptide was conjugated, as evidenced by peptide peak depletion.

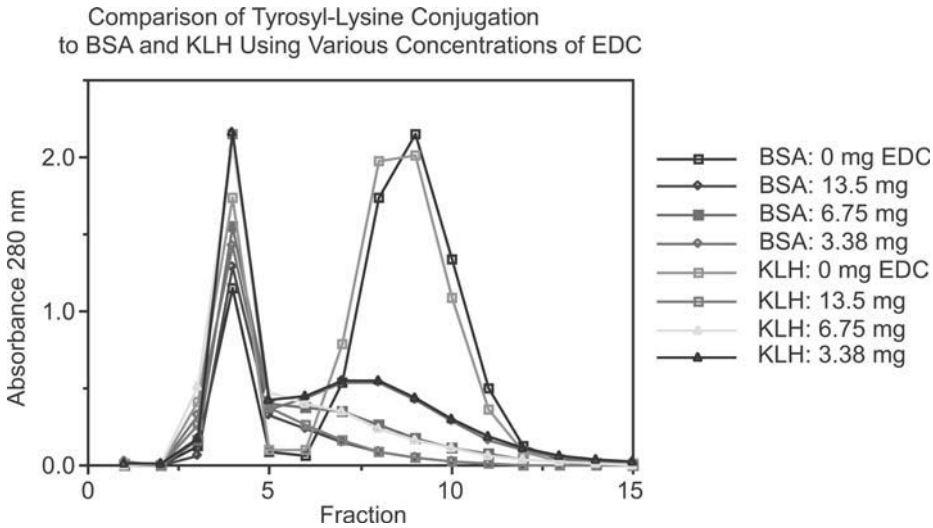

Figure 19.12 EDC conjugation reactions can be extraordinarily consistent using the same peptide crosslinked to two carrier proteins. This figure shows the gel filtration pattern on Sephadex G-25 after completion of the crosslinking reaction. Conjugation of tyrosyl-lysine to BSA and KLH are shown. The first peaks represent eluting carrier, while the second peaks are the excess peptide. Note the consistency of conjugation using the same levels of EDC addition.

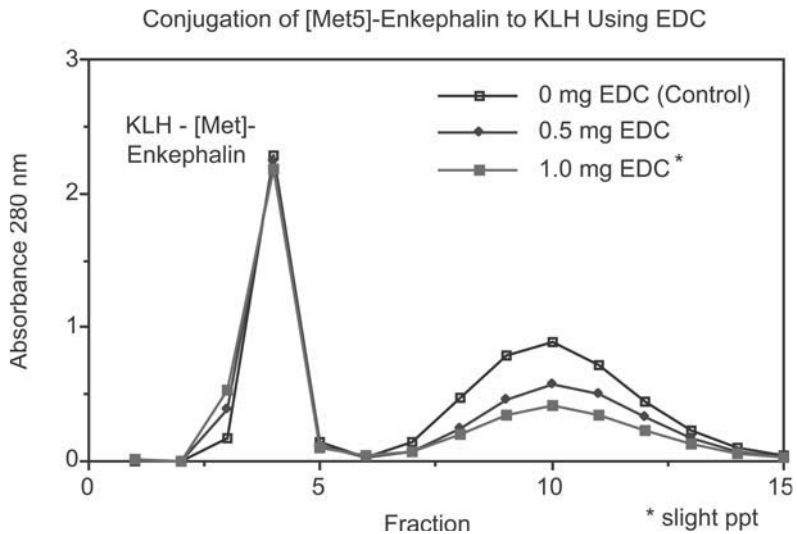

Figure 19.13 Conjugation to KLH often can cause precipitation due to the high-molecular weight of the carrier protein. The conjugation of [Met5]-enkephalin to KLH yields a soluble immunogen if the level of EDC addition is about 0.1 times that typically used with BSA as a carrier. This figure shows the gel filtration pattern on Sephadex G-25 after completion of the crosslinking reaction. The first peak is KLH and the second peak is excess peptide. Depletion of the peptide peak correlates to hapten–carrier conjugation.

resulting from the gel filtration separation of KLH and the peptide [Met5]-enkephalin after an EDC reaction. To result in a soluble immunogen, only 0.1 to 0.2 times the amount of EDC was added as compared to similar BSA or OVA conjugation reactions. Even at this low levels, however, the coupling of peptide to the carrier is very efficient and results in an excellent immunogen.

These studies using EDC-facilitated conjugations were done to develop an optimal protocol for the preparation of immunogens by carbodiimide crosslinking. For haptens (i.e., peptides) that display good solubility in aqueous solution, the level of reagent addition should result in a soluble immunogen conjugate. When using haptens that are sparingly soluble or insoluble in aqueous environments, the conjugation reaction may result in a precipitated complex. Precipitation often can be controlled by scaling back the level of EDC addition or limiting the time of the reaction. If a precipitated immunogen is not a problem (most precipitated, high-molecular weight conjugates are very immunogenic), then the following protocol is applicable to the great majority of peptide-carrier protein conjugations. Thermo Fisher offers a kit containing all the reagents necessary for an EDC-mediated hapten–carrier conjugation.

Protocol

1. Dissolve the carrier protein in 0.1 M MES, 0.15 M NaCl, pH 4.7, at a concentration of 10 mg/ml. If using native, multi-subunit KLH, increase the NaCl concentration of all buffers to 0.9 M (yes, 0.9 M, not 0.9 percent) to maintain solubility of the protein. If using KLH subunits, the high-salt concentration is not necessary. For neutral pH conjugations, substitute 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, for the MES buffer.
2. Dissolve up to 4 mg of the peptide or hapten to be coupled in 1.0 ml of the reaction buffer chosen in step 1. If the peptide to be coupled is already in solution, it may be used directly if it is in a buffer containing no other amines or carboxylic acids and is at a pH between 4.7 and 7.2.

Note: If an assessment of the degree of peptide coupling is desired, measure the absorbance at 280 nm of the 1.0 ml peptide solution before proceeding to step 3. In some cases, a dilution of the peptide solution may be necessary to keep the absorbance on scale for the spectrophotometer. If the peptide is sparingly soluble in aqueous solution, it may be dissolve in DMSO and an aliquot added to the carrier solution. See the previous discussion on carrier proteins to determine the levels of DMSO compatible with carrier protein solubility. Peptide haptens should be at least 5–7 amino acids in length to obtain suitable immunogenicity and correct specificity of the resulting antibodies produced against them. Many immunogen conjugates use peptides that are slightly longer than the desired epitopic sequence to provide enough size to allow interaction with the components of the immune system.

3. Add 500 µl of the peptide solution to 200 µl of carrier protein. For greater reaction volumes, keep the molar ratio of peptide-to-carrier addition the same and proportionally scale up the amount of EDC added in the next step. If the peptide is initially dissolved in DMSO, much less peptide volume compared to protein volume should be used to maintain solubility (see discussion in step 2).
4. For conjugations using relatively low-molecular weight proteins, such as BSA or OVA, add the peptide/carrier solution to a vial containing 10 mg of EDC (Thermo Fisher) and gently mix to dissolve. For high-molecular weight KLH immunogens, first dissolve one

vial containing 10 mg of EDC in 1 ml of deionized water, and immediately transfer 50 µl of this solution to the carrier/peptide solution. Gently mix.

5. Allow the reaction to continue at room temperature for 2 hours.

Note: Although the conjugation protocols have been optimized by preparing a number of different peptide-carrier conjugates, some peptide sequences or other haptens may cause precipitation of the carrier upon coupling. This may occur as a result of changing the carrier's solubility characteristics through surface modification or due to polymerization. A small amount of precipitation is not a problem and can easily be removed by centrifugation before the gel filtration step. If severe precipitation occurs, however, the amount of EDC added to the reaction may have to be scaled back to eliminate or reduce it. With BSA or OVA conjugates, this may mean using as little as 1–3 mg of EDC instead of the recommended 10 mg. With native, multisubunit KLH as a carrier, reducing the EDC levels to 0.1 mg may be necessary.

6. Purify the hapten–carrier conjugate by gel filtration or dialysis.

4. NHS Ester-Mediated Hapten–Carrier Conjugation

Hapten–carrier conjugation may be accomplished by the use of homobifunctional reagents containing NHS ester groups on both ends. The active esters are highly reactive toward amines on proteins and other molecules to form stable amide bonds. Crosslinking agents of various lengths may be used for this conjugation strategy, including the sulfo-NHS ester analogs which are more water-soluble than the NHS esters without a sulfonic acid group (Chapter 4, Section 1).

Using homobifunctional NHS esters, amine-containing haptens may be conjugated to amine-containing carriers in a single step (Figure 19.14). The carrier is dissolved in a buffer having a pH of 7–9 (0.1 M sodium phosphate, pH 7.2 works well). The hapten molecule is added to this solution at a suitable molar excess to assure multipoint attachment of the hapten to the carrier. A molar excess of 20–30 times that of the carrier concentration is a good starting point. Next, the NHS ester crosslinker is added to the solution to provide at least a 3-fold molar excess over that of the hapten. For crosslinkers insoluble in aqueous solution, first solubilize them in dimethylformamide (DMF) or DMSO at higher concentration, and then add an aliquot of this stock solution to the hapten–carrier solution. The conjugation reaction is complete within 2 hours at room temperature. Some adjustment of the level of hapten and crosslinker addition may be necessary to avoid extensive precipitation of the conjugate, especially when using rather hydrophobic hapten molecules.

Another method of NHS ester-mediated hapten–carrier conjugation is to create reactive sulfo-NHS esters directly on the carboxylates of the carrier protein using the EDC/sulfo-NHS reaction described in Chapter 3, Section 1.2. A carbodiimide reaction in the presence of sulfo-NHS activates the carboxylate groups on the carrier protein to form amine-reactive sulfo-NHS esters. The activation reaction is done at pH 6.0, since the amines on the protein will be protonated and therefore be less reactive toward the sulfo-NHS esters that are formed. In addition, the hydrolysis rate of the esters is dramatically slower at acid pH. Thus, the active species may be isolated in a reasonable time frame without significant loss in conjugation potential. To quench unreacted EDC, 2-mercaptoethanol is added to form a stable complex with the remaining carbodiimide, according to Carraway and Triplett (1970). In the following protocol, a modification of Grabarek and Gergely's (1990) two-step method, sulfo-NHS is used instead of NHS

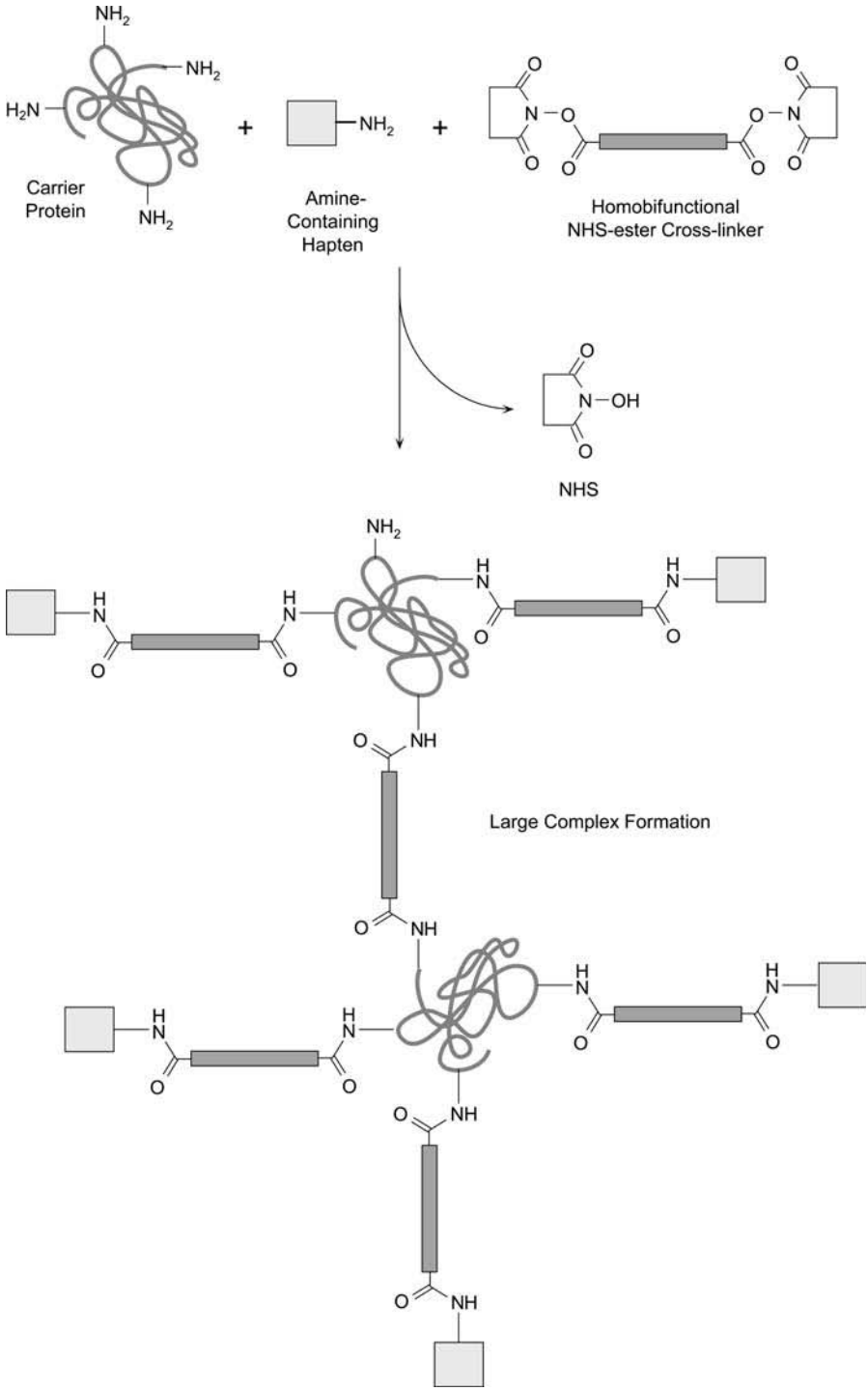

Figure 19.14 Hapten–carrier immunogen conjugates can be formed using homobifunctional NHS ester crosslinkers. The reaction may create large polymeric complexes, some of which could precipitate.

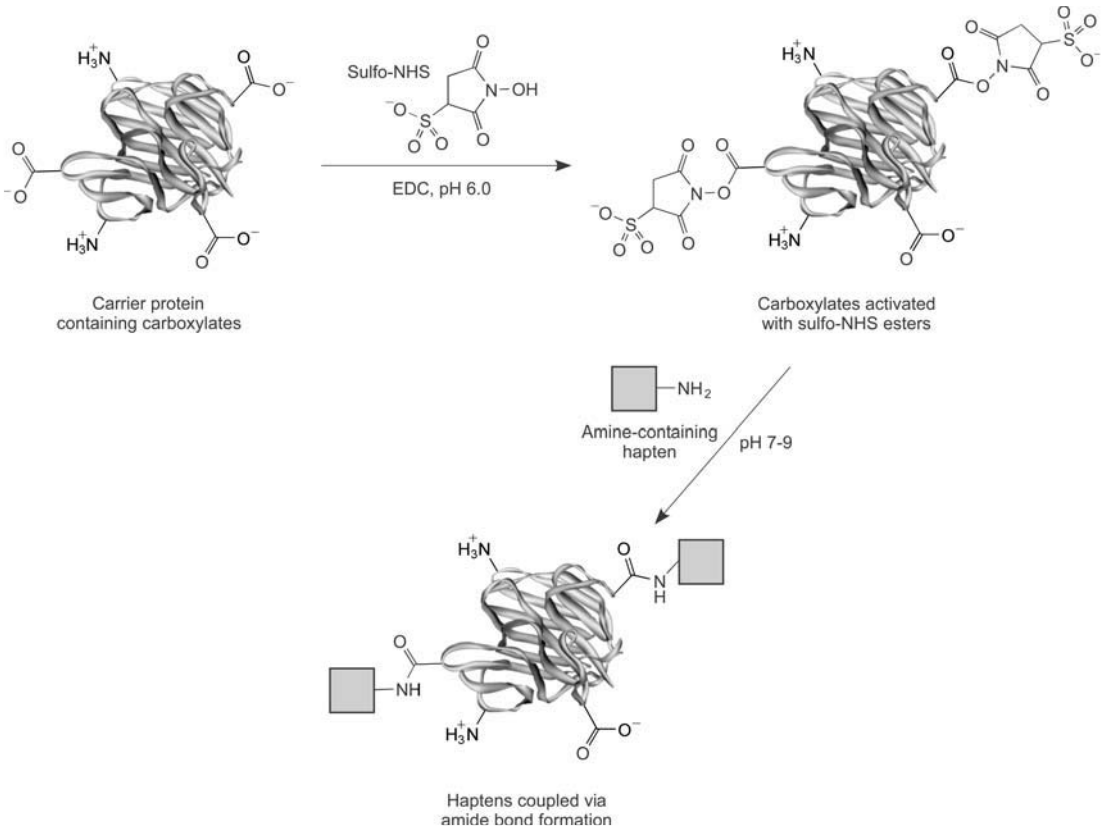

Figure 19.15 The carbodiimide EDC can be used in the presence of sulfo-NHS to create reactive sulfo-NHS ester groups on a carrier protein. Subsequent coupling with an amine-containing hapten can be done to create amide bond linkages.

so that active ester hydrolysis is slowed even more (Anjaneyulu and Staros, 1987; Thelen and Deuticke, 1988). Subsequent conjugation with amine-containing hapten molecules yields hapten–carrier conjugates created by amide bond formation (Figure 19.15).

Protocol

1. Dissolve the carrier protein to be activated in 0.05 M MES, 0.5 M NaCl, pH 6.0 (reaction buffer), at a concentration of 1 mg/ml.
2. Add to the solution in step 1 a quantity of EDC and sulfo-NHS (both from Thermo Fisher) to obtain a concentration of 2 mM EDC and 5 mM sulfo-NHS. To aid in aliquoting the correct amount of these reagents, they may be quickly dissolved in water at a higher concentration, and then immediately a volume pipetted into the protein solution to obtain the proper molar quantities.
3. Mix and react for 15 min at room temperature to form the sulfo-NHS esters.
4. Add 2-mercaptoethanol to the reaction solution to obtain a final concentration of 20 mM. Mix and incubate for 10 min at room temperature.

Note: if the protein being activated is sensitive to this level of 2-mercaptoethanol, instead of quenching the reaction chemically, the activation may be terminated by rapid desalting (see step 5).

5. If the reaction was quenched by the addition of 2-mercaptoethanol, the activated protein may be added directly to an amine-containing hapten molecule for conjugation. Alternatively, or if no 2-mercaptoethanol was added, the activated protein may be purified from reaction by-products by gel filtration using a desalting resin. The desalting operation should be done rapidly to minimize hydrolysis and recover as much active ester functionality as possible. The use of centrifugal spin columns may afford the greatest speed in separation (Thermo Fisher). After purification, add the activated protein to the hapten for conjugation. The hapten molecule should be dissolved in 0.1 M sodium phosphate, pH 7.5.
6. React for at least 2 hours at room temperature.
7. Remove excess reactants by gel filtration or dialysis.

5. NHS Ester-Maleimide Heterobifunctional Crosslinker-Mediated Hapten–Carrier Conjugation

A common method for coupling haptens to carrier proteins involves the use of a heterobifunctional crosslinker containing an NHS ester and a maleimide group. This type of crosslinker allows better control over the coupling process than homobifunctional or zero-length conjugation methods by incorporating a 2- or 3-step reaction strategy directed against two different functional targets. In this approach, the carrier protein first is activated with the crosslinker through its amine groups, purified to remove excess reactants, and then crosslinked to a hapten molecule containing a sulfhydryl group. One of the most useful reagents for this conjugation approach is sulfo-SMCC.

The reactions associated with a sulfo-SMCC conjugation are shown in Figure 19.16 (*Note*: sulfo-SMCC is sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate; MW = 436.37) (see Chapter 5, Section 1.3). This crosslinking reagent mediates the conjugation of a carrier protein through its primary amine groups to a peptide or other hapten through sulfhydryl groups. The active *N*-hydroxysulfosuccinimide ester (sulfo-NHS) end of sulfo-SMCC first is reacted with available primary amine groups on the carrier protein. This reaction results in the formation of an amide bond between the protein and the crosslinker with the release of sulfo-NHS as a by-product. The carrier protein is then isolated by gel filtration to remove excess reagents. At this stage, the purified carrier possesses modifications generated by the crosslinker resulting in a number of reactive maleimide groups projecting from its surface. The maleimide portion of sulfo-SMCC is a thiol-reactive group that can be used in a secondary step to conjugate with a free sulfhydryl (i.e., a cysteine residue) on a peptide or other hapten, resulting in a stable thioether bond.

The use of sulfo-SMCC over the other common maleimide-containing crosslinkers such as *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) or succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB) provides the advantages of initial water-solubility during the activation step and increased stability of the maleimide group prior to conjugation with a peptide. The improved stability assures that the majority of the maleimide groups substituted

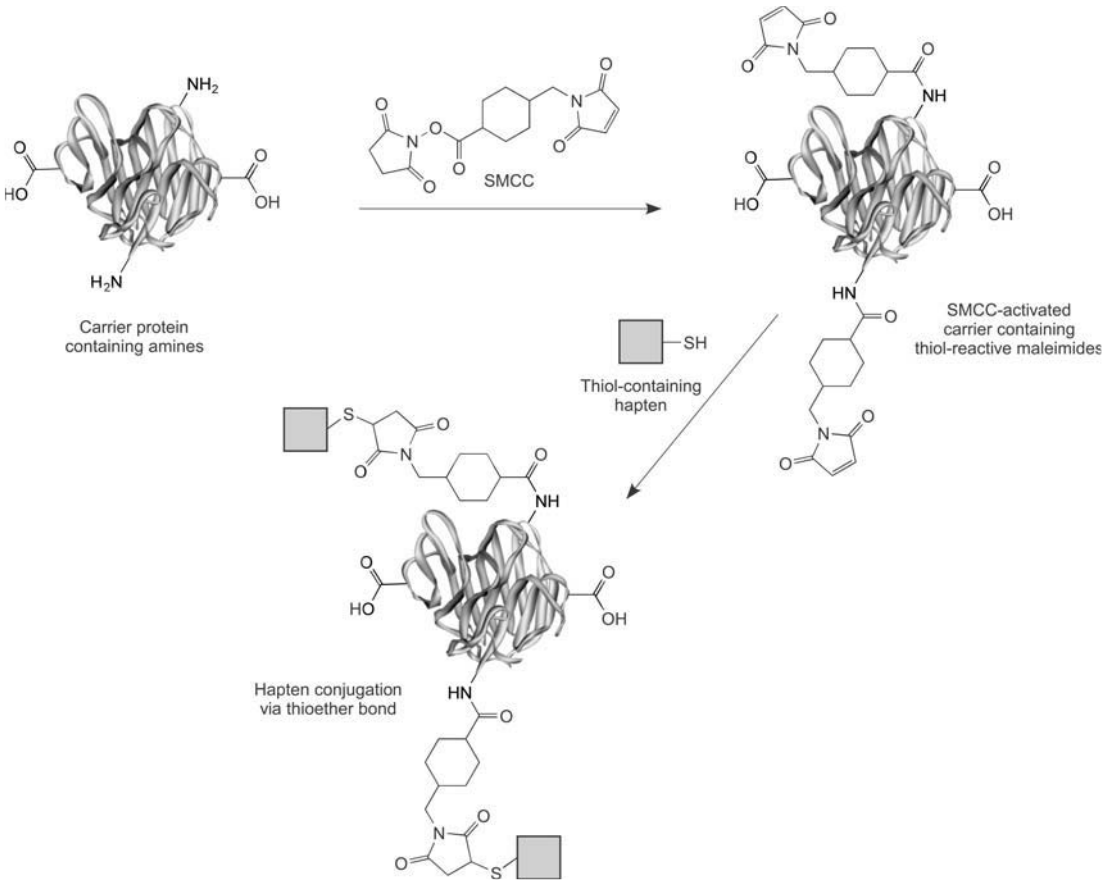

Figure 19.16 A common way of conjugating sulfhydryl-containing haptens to carrier proteins is to activate the carrier with sulfo-SMCC to create an intermediate maleimide derivative. The maleimide groups then can be coupled to thiols to form thioether bonds.

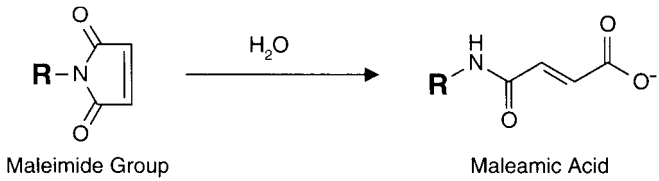

Figure 19.17 A maleimide group may hydrolyze in aqueous solution to an open maleamic acid form that is unreactive with sulfhydryls.

on the carrier will survive the subsequent purification process without degradation. The relatively good stability of the maleimide group of sulfo-SMCC is probably due to the neighboring steric effects of its cyclohexane ring. The faster hydrolysis rates of other maleimide type crosslinkers can be a significant problem, since they readily break down to the maleamic acid form, which is no longer reactive toward sulfhydryls (Figure 19.17).

A disadvantage to using SMCC or other NHS–maleimide type crosslinkers with hindered ring structures (such as MBS) is the relatively high immunogenicity of the cross-bridge. Studies have shown that a hapten–carrier complex formed from such crosslinkers generates significant antibody response against the spacer group itself, not just the hapten and carrier. To minimize the antibody population directed against the cross-bridge of the conjugate, the use of aliphatic straight-chain spacers will exhibit lower immunogenicity (Peeters *et al.*, 1989). However, perhaps a better choice for this type of conjugation is a polyethylene glycol (PEG) based crosslinker containing an NHS ester on one end and a maleimide group on the other end (see Chapter 18, Section 2). A PEG group used as a cross-bridge in a heterobifunctional reagent to prepare immunogen conjugates will result in non-immunogenic modifications on the carrier protein and thus no antibody production against the polyether linker. Although SMCC (or sulfo-SMCC) is used in the following protocol, direct substitution of a PEG-based crosslinker will limit the immune response to the hapten, and not generate unwanted antibodies to the cross-bridge.

Since many peptides do not naturally contain cysteine residues with free sulfhydryls, a terminal cysteine may be incorporated during peptide synthesis, or where appropriate, disulfide groups may be reduced to generate them. Alternatively, thiolating reagents such as 2-iminothiolane (Traut's reagent) can be used to modify existing amino groups and introduce a sulfhydryl (see section 1.1.4.1). Caution must be taken when using this last technique, however, because multiple sites of modification may alter the immunogenic structure of the hapten.

If a terminal cysteine residue is added to a peptide during its synthesis, its sulfhydryl group provides a highly specific conjugation site for reacting with a sulfo-SMCC-activated carrier. All peptide molecules coupled using this approach will display the same basic conformation after conjugation. In other words, they will have a known and predictable orientation, leaving the majority of the molecule free to interact with the immune system. This method therefore can preserve the major epitopes on a peptide while still enhancing the immune response to the hapten by being covalently linked to a larger carrier protein. In addition, the well-known chemical reactivity of a sulfo-SMCC-mediated immunogen preparation permits covalent conjugation in a controllable fashion that can be highly defined for quality assurance purposes.

The process of carrier activation by sulfo-SMCC may be followed by performing a simple purification step after the reaction using a desalting resin. Figure 19.18 shows the gel filtration profiles for the separation of sulfo-SMCC-activated BSA and OVA. The first peak of both separations represents the elution point for the carrier protein, while the absorbance due to reaction by-products of the crosslinker is contained in the second peak (shown only as its leading edge). Activated proteins exhibit an increase in their absorbance at 280 nm over an identical sample with no added sulfo-SMCC due to their covalently attached maleimide groups. After isolation, the activated protein may be frozen and lyophilized to preserve maleimide coupling activity toward sulfhydryl-containing haptens. Thermo Fisher sells a number of maleimide-activated carrier proteins in lyophilized form for easy hapten conjugation.

After a carrier protein has been activated with sulfo-SMCC, it is often useful to measure the degree of maleimide incorporation prior to coupling an expensive hapten. Ellman's reagent may be used in an indirect method to assess the level of maleimide activity of sulfo-SMCC-activated proteins and other carriers. First, a sulfhydryl-containing compound such as 2-mercaptoethanol or cysteine is reacted in excess with the activated protein. The amount of unreacted sulfhydryls remaining in solution is then determined using the Ellman's reaction (Chapter 1, Section 4.1). Comparison of the response of the sample to a blank reaction using

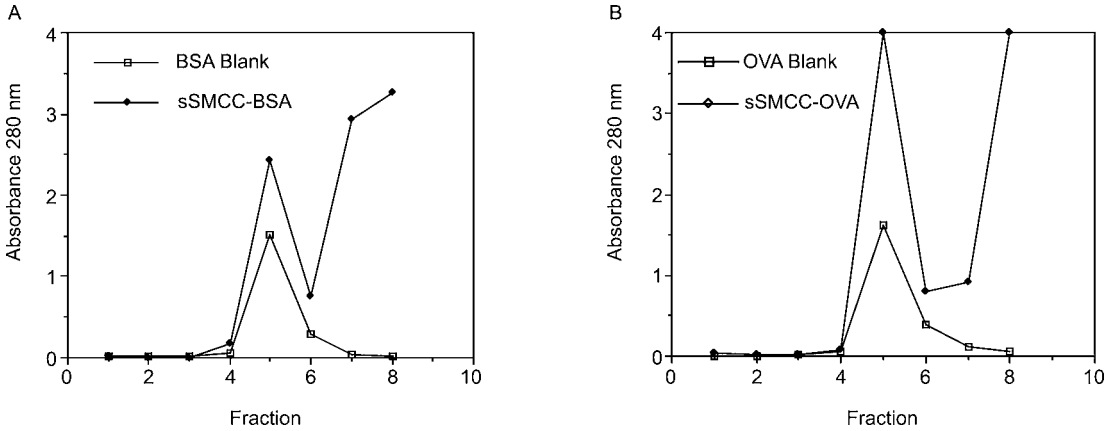

Figure 19.18 Carrier proteins may be activated with sulfo-SMCC to produce maleimide derivatives reactive with sulfhydryl-containing molecules. The graphs show the gel filtration separation on Sephadex G-25 of maleimide-activated BSA (A) and OVA (B) after reaction with sulfo-SMCC. The first peak is the protein and the second peak is excess crosslinker. The maleimide groups create increased absorbance at 280 nm in the activated proteins.

the native, non-activated protein at the same concentration and a series of standards made from a serial dilution of the sulfhydryl compound employed in the assay gives the amount of sulfhydryl compound conjugated and thus an estimate of the original maleimide activity.

Figure 19.19 shows a plot of the results of such an assay done to determine the maleimide content of activated BSA. This particular assay used 2-mercaptoethanol which is relatively unaffected by metal-catalyzed oxidation. For the use of cysteine or cysteine-containing peptides in the assay, however, the addition of EDTA is required to prevent disulfide formation. Without the presence of EDTA at 0.1 M, the metal contamination of some proteins (especially serum proteins such as BSA) is so great that disulfide formation proceeds preferential to maleimide coupling. Figure 19.20 shows a similar assay for maleimide-activated BSA using the more innocuous cysteine as the sulfhydryl-containing compound.

Using this type of cysteine-uptake assay, it is possible to determine the percentage of maleimides that reacted over time. Thus, an indication of the reaction efficiency of a sulfhydryl-containing compound coupling with a maleimide-activated protein may be determined. Figure 19.21 shows the reaction rate for the coupling of cysteine to maleimide-activated BSA. Note that maximal coupling is obtained in less than 2 hours, and over 80 percent yield is achieved in less than 30 minutes.

The following protocol describes the activation of a carrier molecule with sulfo-SMCC and its subsequent conjugation with a hapten. The preactivated carriers containing maleimide groups ready for coupling to a sulfhydryl-containing compound are commercially available in a stable freeze-dried form (Thermo Fisher). Substitution of GMBS (Chapter 5, Section 1.7) or an NHS-PEG-maleimide crosslinker (Chapter 18, Section 2) in the following protocol will provide straight-chain spacer arms with lower or no immunogenicity compared to the ring structure of SMCC's cross-bridge.

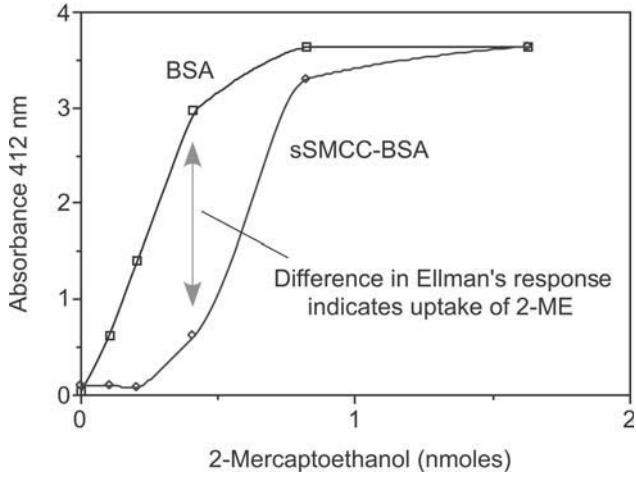

Figure 19.19 An Ellman's assay may be done to determine the maleimide activation level of SMCC-derivatized proteins. Reaction of the activated carrier with different amounts of 2-mercaptoethanol results in various levels of sulfhydryls remaining after the reaction. Detection of the remaining thiols using an Ellman's assay indirectly indicates the amount of sulfhydryl uptake into the activated carrier. Comparison of the Ellman's response to the same quantity of 2-mercaptoethanol plus an unactivated carrier indicates the absolute amount of sulfhydryl that reacted. Calculation of the maleimide activation level then can be done.

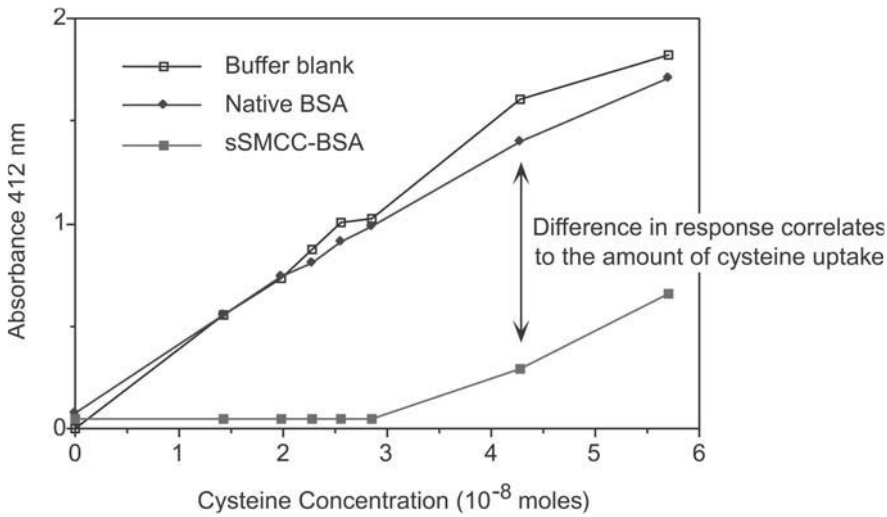

Figure 19.20 Cysteine also may be used in an Ellman's assay to determine the maleimide activation level of SMCC-derivatized proteins. Reaction of the activated carrier with different amounts of cysteine results in various levels of sulfhydryls remaining after the reaction. The coupling must be done in the presence of EDTA to prevent metal-catalyzed oxidation of sulfhydryls. Detection of the remaining thiols using an Ellman's assay indirectly indicates the amount of sulfhydryl uptake into the activated carrier. Comparison of the Ellman's response to the same quantity of cysteine plus an unactivated carrier indicates the absolute amount of sulfhydryl that reacted. Calculation of the maleimide activation level then can be done.

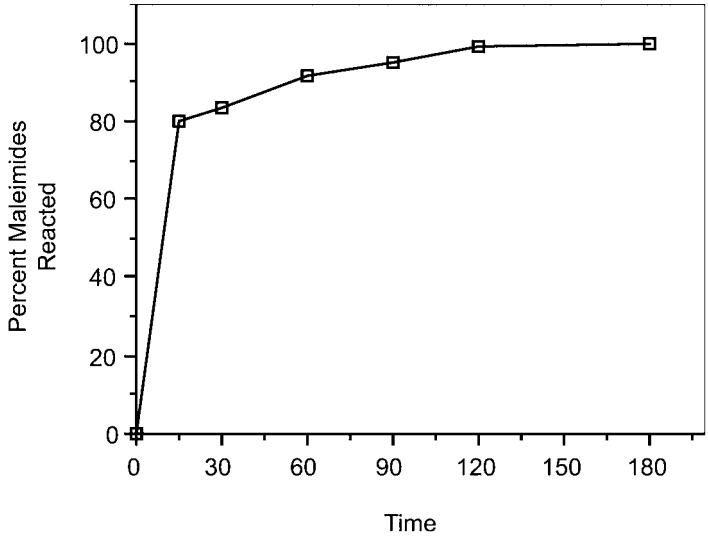

Figure 19.21 The rate of reaction of cysteine with maleimide-activated BSA was determined using an Ellman's assay for remaining sulfhydryl groups after the reaction, according to Figure 19.20. Nearly all of the available maleimides are coupled with sulfhydryls within 2 hour.

Protocol

1. Dissolve the carrier of choice at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2–7.5 (activation buffer).

Note: For use of native, multi-subunit KLH, increase the NaCl concentration to 0.9 M.

2. Dissolve sulfo-SMCC (Thermo Fisher) at a concentration of 10 mg/ml in the activation buffer. Immediately transfer the appropriate amount of this crosslinker solution to the vial containing the dissolved carrier protein.

Note: The amount of crosslinker solution to be transferred is dependent on the level of activation desired. Suitable activation levels can be obtained for the following proteins by adding the indicated quantities of the sulfo-SMCC solution. The degree of sulfo-SMCC modification often determines whether the carrier will maintain solubility after activation and coupling to a hapten. Multimeric KLH in particular, is sensitive to the amount of crosslinker addition. KLH usually retains solubility at about 0.1–0.2 times the mass of crosslinker added to BSA. This level of addition still results in excellent activation yields, since KLH is significantly larger than most of the other protein carriers.

Add the following quantities of sulfo-SMCC solution to each ml of carrier protein solution:

(a) BSA: 500 µl
(b) cBSA: 200 µl
(c) OVA: 500 µl
(d) KLH: 100 µl

Carriers having similar molecular weights to that of BSA or OVA may be activated at the same level with good success. cBSA requires less crosslinker addition due to its greater quantity of amines present.

3. React for 1 hour at room temperature.
4. Immediately purify the activated carrier protein by gel filtration using a desalting resin with a bed volume equal to 15 times the volume of the activation reaction. To perform the chromatography use 0.1 M sodium phosphate, 0.15 M NaCl (0.9 M for KLH), 0.1 M EDTA, pH 7.2–7.5 (conjugation buffer). The EDTA is present to prevent metal-catalyzed sulfhydryl oxidation to disulfides, which will result in an inability to couple to the maleimide groups on the carrier. This is a particular problem when using BSA due to contaminating iron from hemolysis. Concentrations less than 0.1 M EDTA will not fully inhibit the oxidation reaction, especially if a cysteine-containing peptide is to be conjugated to the activated carrier. Apply the sSMCC/carrier reaction mixture to the column while collecting 0.5–1.0 ml fractions. Pool the fractions containing the activated carrier (the first peak to elute from the column), and discard the fractions containing excess sulfo-SMCC (the second peak). The activated carrier should be used immediately or freeze dried to maintain maleimide stability.
5. Dissolve a sulfhydryl-containing hapten or peptide to be conjugated at a concentration of 10 mg/ml in the conjugation buffer. Other hapten concentrations may be used depending on its solubility. If an excess of the peptide solution is made at this time, an estimate of the degree of conjugation may be determined later (see section below). Add this solution to the pooled fractions containing the activated carrier at an equivalent mass ratio (1 mg hapten per mg of the carrier). Alternatively, the peptide may be added in solid form directly to the activated carrier solution if it is known to be freely soluble and can be weighed out in the appropriate quantity.
6. Allow the conjugation reaction to proceed for 2 hours at room temperature.
7. The hapten–carrier conjugate now may be mixed with adjuvant and used for injection purposes without further purification.

An estimate of the degree of conjugation may be made by assaying the amount of sulfhydryl present before and after the coupling reaction. A portion of the peptide solution before mixing with the activated carrier should be saved to compare with the reaction mixture after the conjugation is complete. The comparison is made using a solution of Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid), which reacts with sulfhydryls to form a highly colored chromophore having an absorbance maximum at 412 nm ($\varepsilon_{412\,nm} \times 10^4/cm^{-1}M^{-1}$) (Chapter 1, Section 4.1). A generalized procedure is presented here. Modifications to this guideline may have to be made for each individual peptide to obtain the appropriate response to the Ellman's reagent. For reactions done with very small quantities of peptide, the Ellman's assay may not be sensitive enough to measure the degree of conjugation.

1. Using a microtiter plate (96 well) dispense 200 µl of 0.1 M sodium phosphate, 0.15 M NaCl, 0.1 M EDTA, pH 7.2 (conjugation buffer) into each well to be used.
2. Add 10 µl of the peptide solution before conjugation to the appropriate wells in duplicate.
3. Add 10 µl of the reaction mixture after the conjugation reaction is complete to another set of wells in duplicate.

4. Add 20 µl of Ellman's reagent (1 mg/ml dissolved in the gel filtration purification buffer) to each well including one containing only buffer (220 µl) to use as a blank.
5. Incubate for 15 minutes at room temperature.
6. Measure the absorbance of all wells using a microplate reader with a filter set at 410 nm.

A comparison of the blank corrected values before and after conjugation should give an indication of the percent of peptide coupled. To be more quantitative, a standard curve must be run to focus in on the linear response range of the peptide-Ellman's reaction. Using cysteine as a representative sulfhydryl compound (similar in Ellman's response to a peptide having one free sulfhydryl), it is possible to obtain very accurate determinations of the amount which coupled to the activated carrier. Figure 19.20, discussed previously in this section, shows the results of this type of assay.

6. Active-Hydrogen-Mediated Hapten–Carrier Conjugation

Conjugation chemistry for the coupling of haptens to carrier molecules is fairly well defined for compounds having common functional groups to facilitate such attachment. The types of functional groups generally useful for this operation include easily reactive components such as primary amines, carboxylic acids, aldehydes, or sulfhydryls.

However, for hapten molecules containing no easily reactive functional groups, conjugation can be difficult or impossible using current technologies. To solve this problem, demanding organic synthesis is frequently required to modify the hapten molecule to contain a suitable reactive portion. Particularly, certain drugs, steroidal compounds, dyes, or other organic molecules often have structures that contain no available "handles" for convenient crosslinking.

Frequently, these difficult-to-conjugate compounds do have certain sufficiently active hydrogens that can be reacted with a carrier molecule using specialized reactions designed for this purpose. This section describes two choices for this conjugation problem, the diazonium procedure and the Mannich reaction. Both of them are able to crosslink haptens through any available active hydrogen to carrier molecules, resulting in immunogens suitable for injection.

6.1. Diazonium Conjugation

Diazonium coupling procedures have been used for many years in organic synthesis and for crosslinking or immobilization of active-hydrogen-containing compounds (Inman and Dintzis, 1969; Cuatrecasas, 1970). Diazonium derivatives can couple with haptens containing available phenolic or, to a lesser extent, imidazole groups in an electrophilic substitution reaction (Riordan and Vallee, 1972). They also may undergo minor secondary reactions with sulfhydryl groups and primary amines (Chan *et al.*, 1975).

The most important reaction of a diazonium group, however, is with available tyrosine and histidine residues within peptide haptens, rapidly creating diazo linkages. This method of conjugation is especially useful for site-directed crosslinking of tyrosine-containing peptides. Since tyrosine usually is present only in limited quantities in a given peptide, use of diazonium conjugation can crosslink and orient all peptide molecules in an identical fashion on a carrier. The result is excellent reproducibility in preparation of the immunogen, and a consistent presentation of the peptide on the surface of the carrier to the immune system for antibody production.

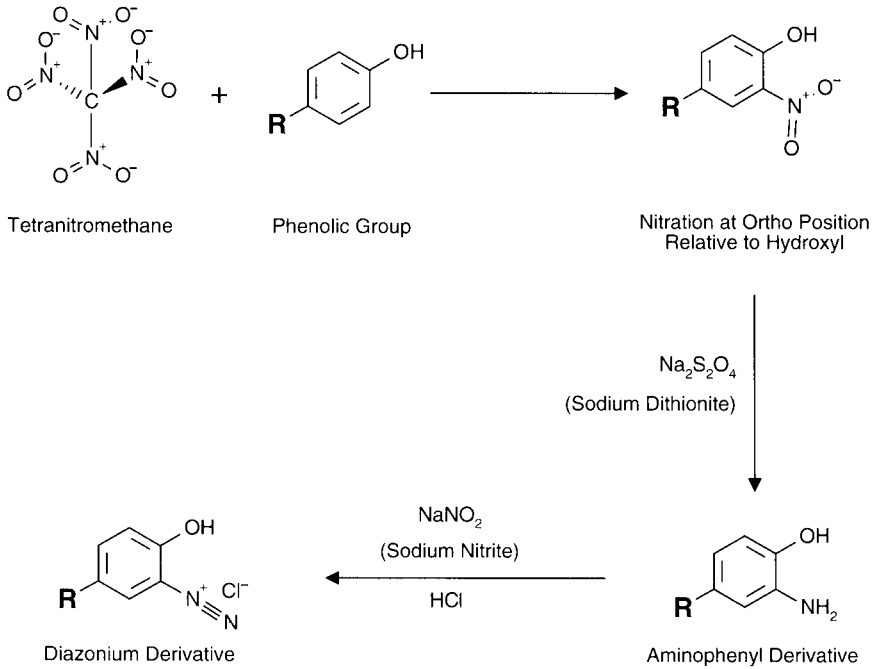

Figure 19.22 Phenolic compounds may be derivatized to contain reactive diazonium groups by nitration with tetranitromethane followed by reduction with sodium dithionite and diazotization with sodium nitrite in dilute HCl.

Derivatives of carbohydrate antigens also have been coupled to carrier proteins through the use of an intermediate diazonium group (McBroom *et al.*, 1976). In this case, an aminophenyl glycoside was prepared by reaction of the reducing end of the oligosaccharide with β-(*p*-aminophenyl)ethylamine and then forming the diazotized derivative with sodium nitrite (Zopf *et al.*, 1978). Upon mixing with carrier proteins containing tyrosine residues, the carbohydrate derivative is coupled via a diazo bond.

Creation of a diazonium group on phenolic compounds or tyrosine side-chain groups is possible by forming an intermediate nitrophenol derivative. Reaction of tyrosine-containing proteins and peptides with tetranitromethane effectively nitrates the ring in the *ortho* position (Vincent *et al.*, 1970). Reduction of the nitro group to an amine then is done using sodium dithionite (sodium hydrosulfite; $Na_2S_2O_4$) (Sokolovsky *et al.*, 1967; Chapter 1, Section 4.3). The aminophenol derivative finally is reacted with sodium nitrite in acidic conditions to form the highly reactive diazonium group (Figure 19.22). Once created, the diazonium compound must be added immediately to the conjugation reaction, since the species is extremely unstable in aqueous environments.

The active diazonium typically is a colored compound, sometimes orange, dark brown, or even black in concentrated solutions. The conjugated immunogen usually is deeply colored due to the resultant diazo bond. The coupling reaction is performed at alkaline pH, optimally at pH 8 for histidine residues and pH 9–10 for tyrosine groups. In practice, however, it is not possible to target a histidine group in the presence of a tyrosine group without some cross-reactivity.

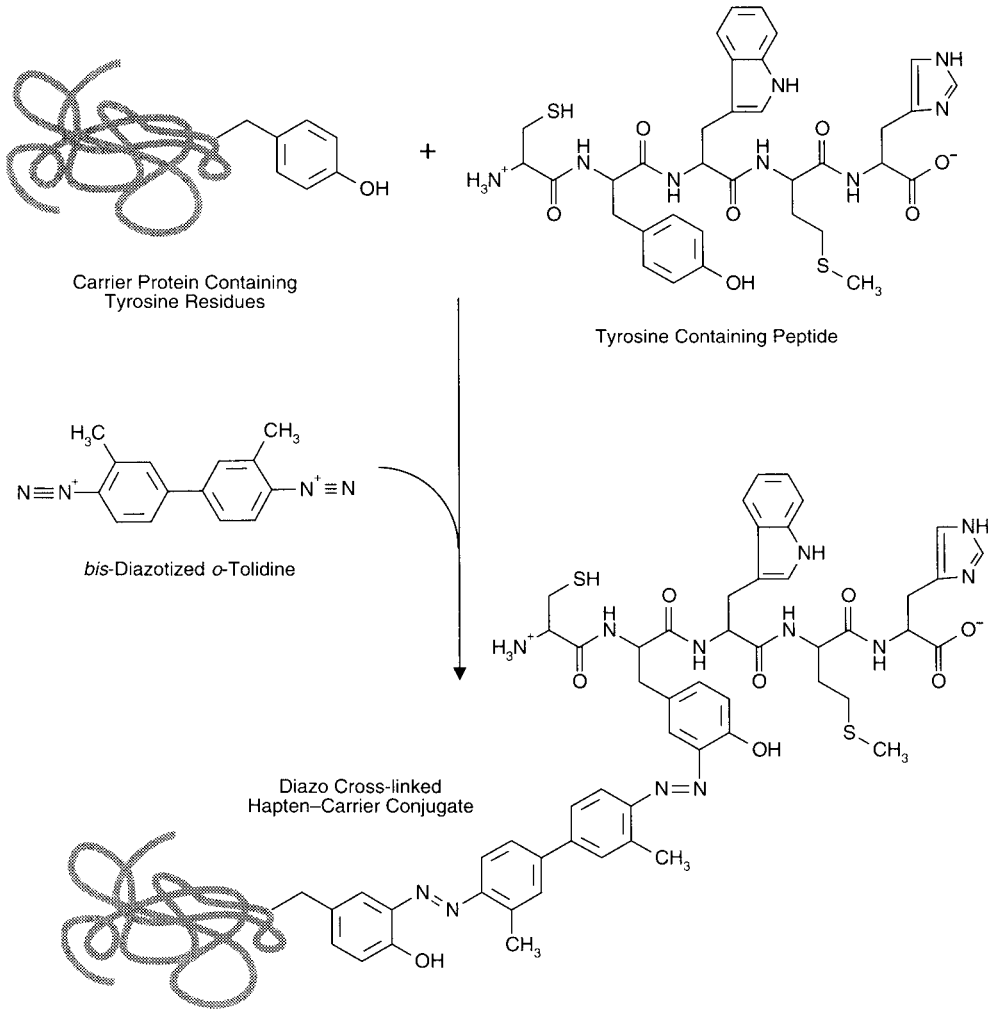

Figure 19.23 The conjugation of a tyrosine-containing carrier protein and a tyrosine-containing peptide may be done using bis-diazotized tolidine to form diazo crosslinks.

Diazo linkages are reversible bonds that may be cleaved by addition of 0.1 M sodium dithionite in 0.2 M sodium borate, pH 9. Release of the crosslinks can be followed by loss of the diazo bond color.

A simple, one-step conjugation reaction is possible with diazonium chemistry if a *bis*-aminophenyl compound is used as a homobifunctional crosslinking agent. Activation of the aminophenyl groups with sodium nitrite creates the requisite *bis*-diazonium derivative that then can couple with active-hydrogen-containing haptens and carriers. In this way, tyrosine-containing peptides can be conjugated with tyrosine-containing carrier proteins in a single step. Compounds useful for this procedure include *o*-tolidine and benzidine (Chapter 4, Section 9), both of which contain aromatic amines that easily can be diazotized (Figure 19.23).

From a practical perspective, however, any of the conjugation methods utilizing diazonium chemistry can be problematic. The rate of reaction of the diazonium species is so rapid that much of the total coupling potential can be lost through intramolecular crosslinking. As the diazonium groups are formed they may immediately crosslink to the active hydrogens present on the aminophenyl precursor molecules, even before addition of a second molecule to be conjugated. Even without addition of a second active-hydrogen-containing compound, the diazonium activated molecule will turn brown to black within an hour, indicating formation of diazo bonds and self-conjugation. For this reason, the reproducibility of conjugation reactions using this method can be poor.

The following protocol describes the use of diazotized *o*-tolidine for the crosslinking of active-hydrogen-containing haptens to active-hydrogen-containing carriers. Using a *bis*-diazonium compound is perhaps the simplest method of conjugation, but as in many one-step crosslinking procedures, it often results in some precipitation of the final product. Reaction conditions may have to be adjusted to prevent severe precipitation, however even an insoluble immunogen can be useful in generating an antibody response.

Caution: Both *o*-tolidine and benzidine are potential carcinogens. Protective clothing, gloves, and the use of a fume hood are recommended. Avoid all contact of the compounds with skin or clothing and do not inhale vapors or dust.

Protocol

1. Diazotization of *o*-tolidine: Weigh out 25 mg of *o*-tolidine and place in a small test tube or vial. Add 4.5 ml of 0.2 N HCl and mix to dissolve. Chill the solution on ice. Dissolve 17.5 mg of sodium nitrite into 0.5 ml of ice-cold deionized water, and add it to the vial containing the *o*-tolidine. The solution should begin to turn an orange color, progressively getting darker as the reaction continues. React for 1 hour on ice, mixing periodically. At the completion of the diazotization reaction, aliquots of the solution may be stored at −20°C.
2. Dissolve 10 mg of carrier protein into 0.5 ml 0.15 M sodium borate, 0.15 M NaCl, pH 9.0.
3. Dissolve 5–10 mg of a peptide hapten containing at least one tyrosine residue per ml of 0.15 M sodium borate, 0.15 M NaCl, pH 9.0.
4. Mix 0.5 ml of the peptide solution with 0.5 ml of the carrier protein solution. Chill on ice. Add 0.4 ml of the *bis*-diazotized tolidine solution. There should be a color change from orange to red almost immediately. Continue the reaction for 2 hours on ice in the dark.
5. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4. The preparation is now ready for immunization purposes.

6.2. Mannich Condensation

Another approach for crosslinking haptens to carriers when the hapten has no available common functional groups (amines, carboxylates, sulfhydryls, etc.), but does possess active hydrogens, is to use the Mannich reaction. Using this strategy an active-hydrogen-containing compound can be condensed with formaldehyde and an amine in the Mannich reaction resulting

in a stable alkylamine linkage. Particularly, compounds containing replaceable hydrogens provided by the presence of certain activating chemical constituents can be aminoalkylated using this reaction (see Chapter 2, Section 5.4 and Chapter 4, Section 6.1 for additional information on active hydrogens).

In its simplest form, the Mannich reaction consists of the condensation of formaldehyde (or sometimes another aldehyde) with ammonia, in the form of its salt, and another compound containing an active hydrogen. Instead of using ammonia, however, this reaction can be done with primary or secondary amines, or even with amides. An example is illustrated in the condensation of acetophenone, formaldehyde, and a secondary amine salt (the active hydrogens are shown underlined):

$$C_6H_5COC\underline{H}_3 + CH_2O + R_2NH \cdot HCl \rightarrow C_6H_5COCH_2CH_2NR_2 \cdot HCl + H_2O$$

The Mannich reaction provides a viable alternative to the diazonium conjugation method (discussed previously), because of the disadvantages inherent in the instability of both the diazonium group and the resultant diazo linkage. By contrast, conjugations done through Mannich condensations result in stable covalent bonds.

The crosslinking scheme using this method can make use of the native ε- and N-terminal amines on carrier proteins as the source of primary amine for the condensation reaction. Added to the conjugation reaction then is formaldehyde and the desired hapten to be coupled containing an appropriately active hydrogen.

To increase the yield of conjugated hapten using this procedure, cBSA is used as the carrier protein in the method described below (see Section 2.1, this chapter for additional information on this carrier). The greater density of amine groups on cBSA available for participation in the Mannich reaction over that available on native proteins provides better results in coupling active-hydrogen-containing haptens.

One note of caution should be realized when using the Mannich reaction. The hapten to be coupled should not contain any amine groups or hapten polymerization may occur preferential to conjugation to the carrier. For instance, when performing site-directed coupling of tyrosine-containing peptides through their phenolic side chain, the diazonium reaction should be used instead of the Mannich procedure, otherwise peptide-to-peptide coupling may occur.

Protocol

1. In a vial or test tube the following are placed and mixed:
   a. 200 µl of a solution containing 10 mg/ml cBSA (Thermo Fisher) in 0.1 M MES, 0.15 M NaCl, pH 4.7 (coupling buffer). The acidic conditions of this coupling buffer are optimal for the Mannich reaction.
   b. 200 µl of a solution consisting of 10 mg/ml of a hapten containing an active hydrogen. The solution can be made up in absolute ethanol in the case of water-insoluble haptens and is made up in coupling buffer in the case of water-soluble haptens.
   c. 50 µl of additional absolute ethanol in the case of water-insoluble haptens.
   d. 50 µl of 37 percent formaldehyde (Sigma) solution.

Caution: Use a fume hood and avoid contact or inhalation of vapors.

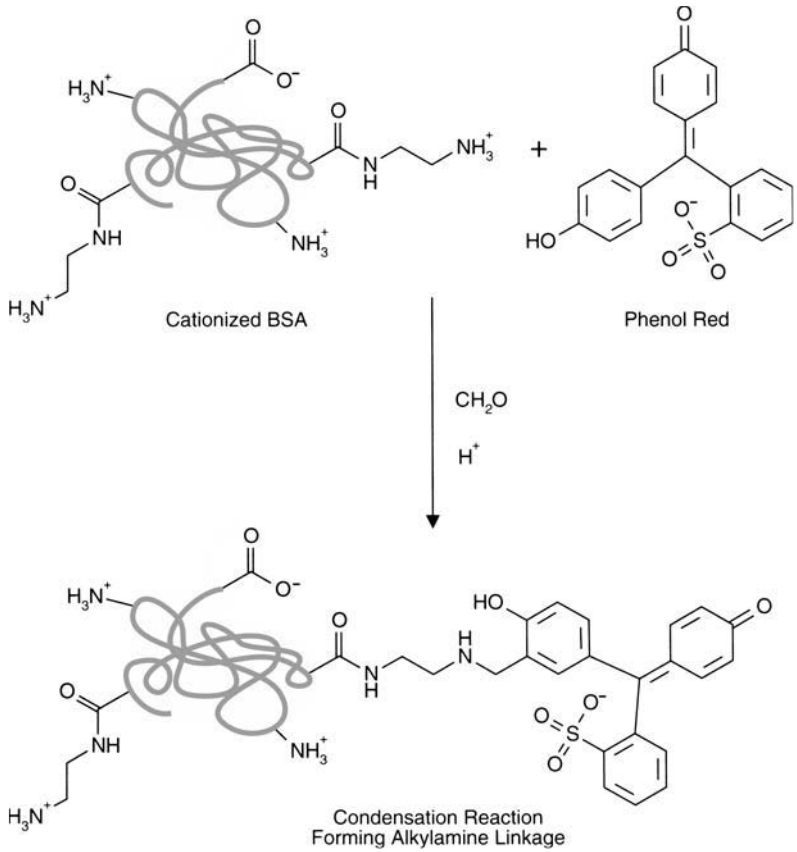

Figure 19.24 The conjugation of phenol red to cBSA using the Mannich reaction.

2. Incubate the reaction mixture in a water bath or oven at a temperature of 37–57°C for a period of 3–24 hours.
3. To separate unconjugated hapten and formaldehyde from the synthesized conjugate, apply the entire volume of reactants to a desalting column containing a bed volume of at least 10 times the volume of the reaction mixture. PBS, pH 7.2 can be used for the desalting step. The purified conjugate is recovered in the void volume.

The yield of conjugation using the Mannich reaction is dependent on the reactivity of active hydrogens within the hapten molecule. It is often difficult to predict the relative reactivity of any given compound in this reaction. Thus, trial and error may be necessary to determine the suitability of the Mannich procedure.

Figure 19.24 shows the conjugation reaction of the dye phenol red to cBSA using the Mannich reaction. The active hydrogens which participate in the conjugation are *ortho* to the hydroxyl group on the phenol ring. After purification of the conjugate by gel filtration to remove any unconjugated dye and formaldehyde, a wavelength scan was done to assess the degree of conjugate formation. Figure 19.25 shows the results of this scan. The protein

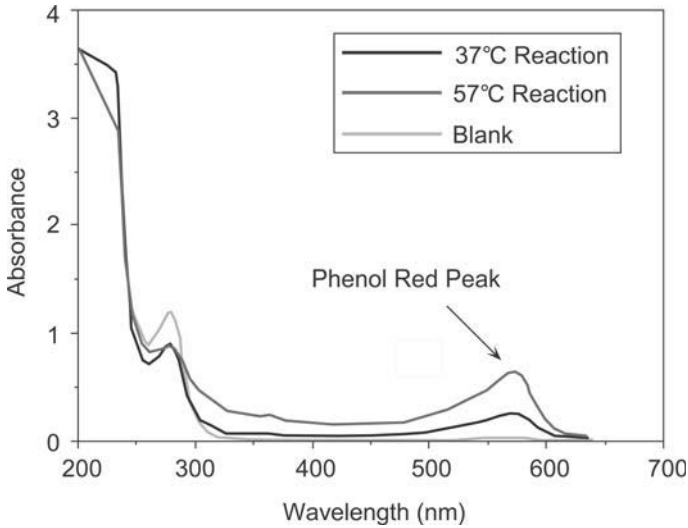

Figure 19.25 Absorbance scan comparing unconjugated cBSA with the same carrier that had been coupled with phenol red using the Mannich reaction. Two different reaction times are compared, indicating that extended reactions yield increased conjugate formation.

solution appeared red after conjugation and desalting, indicating successful crosslinking had occurred.

The steroidal compound 17β-estradiol was also conjugated to cBSA using the Mannich reaction. Similar to phenol red, conjugation with estradiol occurs *ortho* to the hydroxyl group on its phenolic ring (Figure 19.26). After purification of the conjugate by gel filtration, it was injected in mice intraperitoneally using alum as adjuvant. Antibodies were successfully produced against the coupled estradiol. Controls consisting of unconjugated estradiol with and without mixed carrier molecules also were injected, but resulted in no antibody production.

7. Glutaraldehyde-Mediated Hapten–Carrier Conjugation

The homobifunctional crosslinking reagent glutaraldehyde can be used in a one- or two-step conjugation protocol to prepare hapten–carrier conjugates. Glutaraldehyde can react with primary amine groups to create Schiff bases or double bond (Michael-type) addition products (Chapter 4, Section 6.2). The Schiff base intermediate may form resonance-stabilized products with the α,β-unsaturated aldehydes of the glutaraldehyde polymers predominating at basic pH values (Korn *et al*., 1972; Monsan *et al*., 1975; Peters and Richards, 1977). One such product, a quaternary pyridinium complex, can form as a crosslink between two lysine residues (Chapter 1, Section 4.4). Reduction of the Schiff bases with sodium borohydride or sodium cyanoborohydride yields stable secondary amine linkages.

The reaction of glutaraldehyde with protein carriers and peptide haptens involves mainly lysine ε-amine and N-terminal α-amine groups. The conjugates formed are usually of high-molecular weight and may cause precipitation products. In addition, the orientation of the

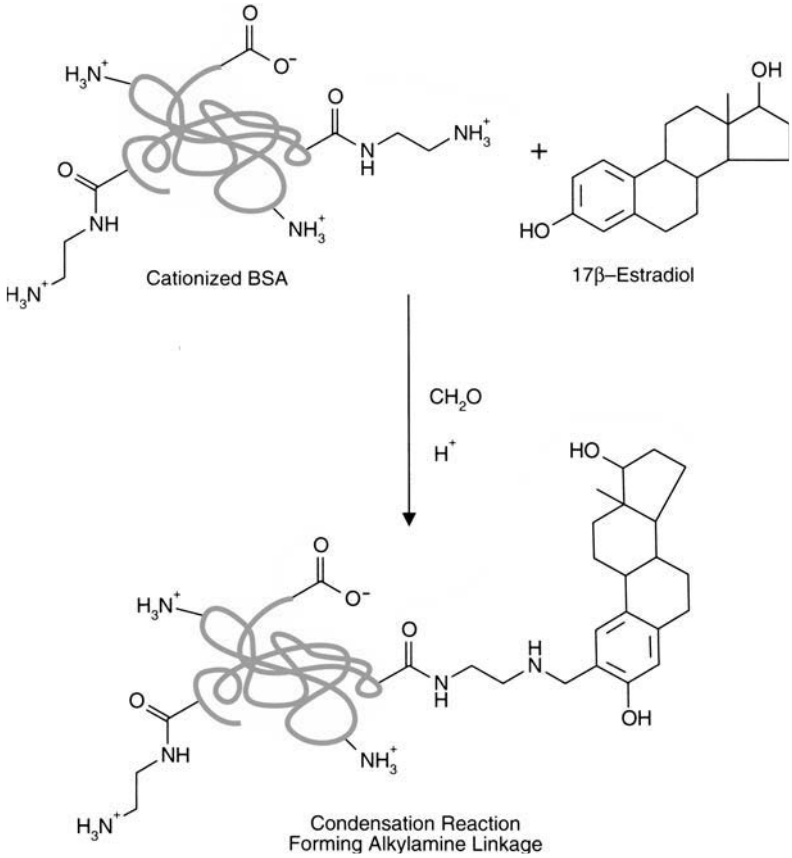

Figure 19.26 The conjugation of estradiol to cBSA using the Mannich reaction.

hapten on the carrier is indiscriminate with oligomers of the peptide predominating. However, despite the disadvantages of using glutaraldehyde-mediated crosslinking, it still remains one of the most popular techniques for creating bioconjugates.

There are several different protocols commonly used in the literature to form glutaraldehyde conjugates. Some methods utilize a neutral pH environment in phosphate buffer (pH 6.8–7.5) while others use more alkaline pH conditions in carbonate buffer (pH 8–9) (Price *et al.*, 1993). In general, the higher pH conditions will more effectively form Schiff base intermediates and result in greater conjugation yields, but also higher-molecular weight conjugates. The concentration of glutaraldehyde in the reaction medium generally varies from 0.20 to 1 percent (Avrameas, 1969; Ford *et al.*, 1978; Jeanson *et al.*, 1988) with occasional use of very dilute solutions (0.05 percent). The lower concentrations of glutaraldehyde generate lower yields of conjugation and result in less stable conjugates (Briand *et al.*, 1985).

The following procedure utilizes the one-step glutaraldehyde method. A two-step method may be used to somewhat limit polymerization of the conjugate (Chapter 20, Section 1.2). Varying the pH and the amount of glutaraldehyde added to the reaction can control the yield and molecular weight of the conjugates formed.

Protocol

1. Dissolve the carrier protein (or another carrier that contains amine groups) in 0.1 M sodium carbonate, 0.15 M NaCl, pH 8.5, at a concentration of 2 mg/ml.
2. Add peptide hapten to the carrier solution to obtain a concentration of about 2 mg/ml. Alternatively, determine the molar ratio of peptide to carrier. Ratios of 20:1 to 40:1 (peptide:carrier) usually result in good immunogens.
3. Add fresh glutaraldehyde to the peptide/carrier solution to obtain a 1 percent final concentration. Mix well.

Caution: Use of a fume hood is recommended when working with glutaraldehyde. Avoid contact with skin and clothing. Do not breathe vapors.

4. React for 2–4 hours at 4°C. Periodically mix the solution or use a gentle rocker.
5. The conjugate may be stabilized by addition of a reductant such as sodium borohydride or sodium cyanoborohydride. Usually sodium cyanoborohydride is recommended for specific reduction of Schiff bases, but since the conjugate has already formed at this point, the use of sodium borohydride will both reduce the associated Schiff bases and eliminate any remaining aldehyde groups. Add sodium borohydride to a final concentration of 10 mg/ml. Continue to react for 1 hour at 4°C.
6. Purify the conjugate to remove excess reagents by gel filtration using a desalting resin or by dialysis. The presence of high-molecular weight conjugates may cause some precipitation in the final product. If turbidity is evident, instead of using gel filtration, dialyze against PBS, pH 7.4.

8. Reductive Amination-Mediated Hapten–Carrier Conjugation

Hapten molecules containing aldehyde residues may be crosslinked to carrier molecules by use of reductive amination (Chapter 3, Section 4). At alkaline pH values, the aldehyde groups form intermediate Schiff bases with available amine groups on the carrier. Reduction of the resultant Schiff bases with sodium cyanoborohydride or sodium borohydride creates a stable conjugate held together by secondary amine bonds.

Oligosaccharide haptens are especially amenable for coupling to carriers by reductive amination. Carbohydrate molecules may contain reducing ends that can be utilized for this purpose (Chapter 1, Section 2.1) (Gray, 1978), or aldehyde residues may be specifically created from other functional groups (Chapter 1, Section 4.4). Often, mild oxidation using sodium periodate can be used to cleave adjacent diols on sugar residues, forming reactive aldehyde groups (Anderson *et al.*, 1989), but this process may alter the antigenic epitopes from that of the native carbohydrate. In addition, the reducing ends of glycans can be coupled to carrier molecules after their release from glycoproteins or other glycoconjugates (Chapter 1, Section 4.6). This technique can be used to create antibodies that are specific for binding the glycosylation sites on certain proteins.

If the reducing ends of oligosaccharide or glycan molecules are used for this technique, then the time necessary to obtain good yields of hapten–carrier conjugates may be from several days to several weeks, depending upon the reaction conditions used. The extended reaction period is due to the limited time-reducing sugars are in their open, aldehydic form (usually far less than

1 percent of the available saccharide at any given time). By contrast, if periodate-oxidized carbohydrate is used, then the reaction time is reduced to only hours. It should be noted, however, that extensive periodate oxidation could modify antigenic determinants and no longer reflect the native structure and characteristics of the carbohydrate.

Protocol

1. Dissolve the carrier protein at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 8.0.
2. Add the aldehyde-containing oligosaccharide to the carrier solution at a concentration sufficient to obtain at least a 20-fold molar excess of hapten to carrier. Adding a much greater molar excess of oligosaccharide to couple through reducing ends (i.e., up to 200-fold excess) will help to drive the conjugation reaction to completion.
3. Add sodium cyanoborohydride (Thermo Fisher) to the mixture to give a final concentration of 20 mg/ml.

Caution: Highly toxic! Use a fume hood and avoid inhalation of dust or vapors. Seal the reaction vessel with parafilm. Do not use a rigid sealing cap, since cyanoborohydride will liberate hydrogen gas bubbles over time and may rupture the vessel.

4. React at room temperature or at 37°C with periodic mixing. Reaction times can vary significantly depending on the reactivity of the aldehyde group. For coupling of the reducing ends of polysaccharide or glycan molecules, continue the reaction for 2–4 days. High density derivatization through the reducing ends may take up to 2 weeks. For coupling of periodate-oxidized carbohydrate, where the aldehyde residues are more accessible, the reaction is complete within 4 hours. See Chapter 1, Section 4.6 for additional protocol options for working with glycans.
5. Purify the hapten–carrier conjugate to remove excess reductant by gel filtration or dialysis using a PBS, pH 7.4 buffer. Removal of unconjugated carbohydrate may be more difficult. If the oligosaccharide was of high-molecular weight so that the unconjugated carbohydrate cannot be easily separated from the conjugate using typical desalting gels or small-porosity dialysis tubing, then a gel filtration matrix possessing greater exclusion limits may be used. However, often it is not necessary to remove unconjugated hapten from such preparations.

20

Antibody Modification and Conjugation

The ability to conjugate an antibody to another protein or molecule is critically important for many applications in life science research, diagnostics, and therapeutics. Antibody conjugates have become one of the most important classes of biological agents associated with targeted therapy for cancer and other diseases. There literally are dozens of markers that have been identified on tumor cells to which monoclonal antibodies have been developed for targeted therapy (Carter *et al.*, 2004). The preparation of antibody conjugates to find and destroy cancer cells *in vivo* has become one of the leading strategies of research into investigational new drugs (McCarron *et al.*, 2005). In most cases, the site-specific delivery of drugs involves the successful development of defined monoclonal antibody conjugates that can target diseased cells without affecting normal ones.

In addition, the use of antibody molecules in immunoassay or detection techniques encompasses a broad variety of applications affecting nearly every field of research. The availability of relatively inexpensive polyclonal and monoclonal antibodies of exacting specificity has made possible the design of reagent systems that can interact in high affinity with virtually any conceivable analyte. The directed specificity of purified immunoglobulins provides powerful tools for constructing immunological reagents. Using a number of conjugation and modification techniques, these specific antibodies can be modified to allow easy tracking in complex mixtures. For instance, an antibody molecule labeled with an enzyme, a fluorescent compound, or biotin provides a detectable complex able to be quantified or visualized through its tag.

To maintain specificity in antibody conjugates derived from polyclonal antisera, only affinity purified immunoglobulins should be used. Such purified preparations are isolated from antisera by affinity chromatography using the corresponding immobilized antigen. These preparations thus contain only that population of antibody molecules which has the desired antigenic specificity. Modification or conjugation of whole immunoglobulin fractions should be avoided, since other antibody populations will be present and cause considerable nonspecificity in the resultant activity of the reagent. Even secondary antibodies should be affinity purified and highly cross-adsorbed against immunoglobulins of other species' antibody types to prevent nonspecific interactions.

Monoclonal antibodies also should be purified by affinity chromatography prior to undergoing bioconjugation. This can be accomplished using an immobilized antigen or, if the antigen is not available in large enough quantities, an immobilized immunoglobulin binding protein

(such as protein A) may be employed. Most monoclonals that can be successfully purified while maintaining activity also will be stable enough to withstand the rigors of chemical modification. Occasionally, however, a particular monoclonal will be partially or completely inactivated through the modification reaction. Sometimes this activity loss is caused by physically blocking the antigen binding sites during conjugation. In other cases, conformational changes in the complementarity-determining regions are the cause of the problem. If the antigen binding site is merely being blocked, then choosing an appropriate site-directed chemistry may solve the problem. On the other hand, some monoclonals are too labile to undergo modification reactions, regardless of the coupling method. Trial and error often is necessary when working with monoclonals to determine if modification will severely affect activity.

The unique structural characteristics of antibody molecules supply a number of choices for modification and conjugation schemes (Roitt, 1977; Goding, 1986; Harlow and Lane, 1988a, b, c). The chemistry used to effect conjugate formation should be chosen to yield the best possible retention of antigen binding activity. A detailed illustration of antibody structure is shown in Figure 20.1. The most basic immunoglobulin G molecule is composed of two light and two heavy chains, held together by noncovalent interactions as well as a number of disulfide bonds. The light chains are disulfide-bonded to the heavy chains in the C_L and C_H1 regions, respectively. The heavy chains are in turn disulfide-bonded to each other in the hinge region.

The heavy chains of each immunoglobulin molecule are identical. Depending on the class of immunoglobulin, the molecular weight of these subunits ranges from about 50,000 to around

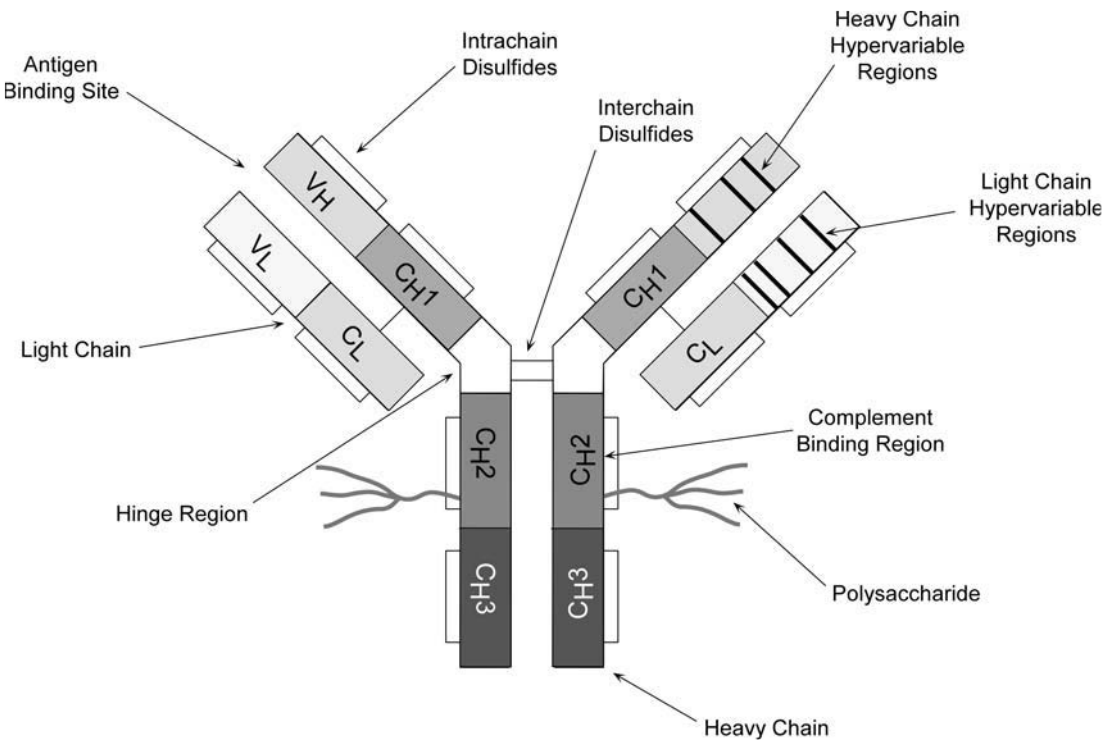

Figure 20.1 Detailed structure of an IgG antibody molecule.

75,000. Similarly, the two light chains of an antibody are identical and have a molecular weight of about 25,000. For IgG molecules, the intact molecular weight representing all four subunits is in the range of 150,000–160,000.

There are two forms of light chains that may be found in antibodies. A single antibody will have light chain subunits of either lambda (λ) or kappa (κ) variety, but not both types in the same molecule. The immunoglobulin class, however, is determined by an antibody's heavy chain variety. A single antibody also will possess only one type of heavy chain (designated as γ, μ, α, ε, or δ). Thus, there are five major classes of antibody molecules, each determined from their heavy chain type, and designated as IgG, IgM, IgA, IgE, or IgD. Three of these antibody classes, IgG, IgE, and IgD, consist of the basic Ig monomeric structure containing two light and two heavy chains. By contrast, IgA molecules can exist as a singlet, doublet, or triplet of this basic Ig monomeric structure, while IgM molecules are large pentameric constructs (Figure 20.2). Both IgA and IgM contain an additional subunit, called the J chain—a very acidic polypeptide of molecular weight 15,000 that is very rich in carbohydrate. The heavy chains of immunoglobulin molecules also are glycosylated, typically in the C_H2 domain within the Fc fragment region, but also may contain carbohydrate near the antigen binding sites.

There are two antigen binding sites on each of the basic Ig-type monomeric structures, formed by the heavy–light chain proximity in the N-terminal, hypervariable region at the tips of the "y" structure. The unique tertiary structure created by these subunit pairings produces

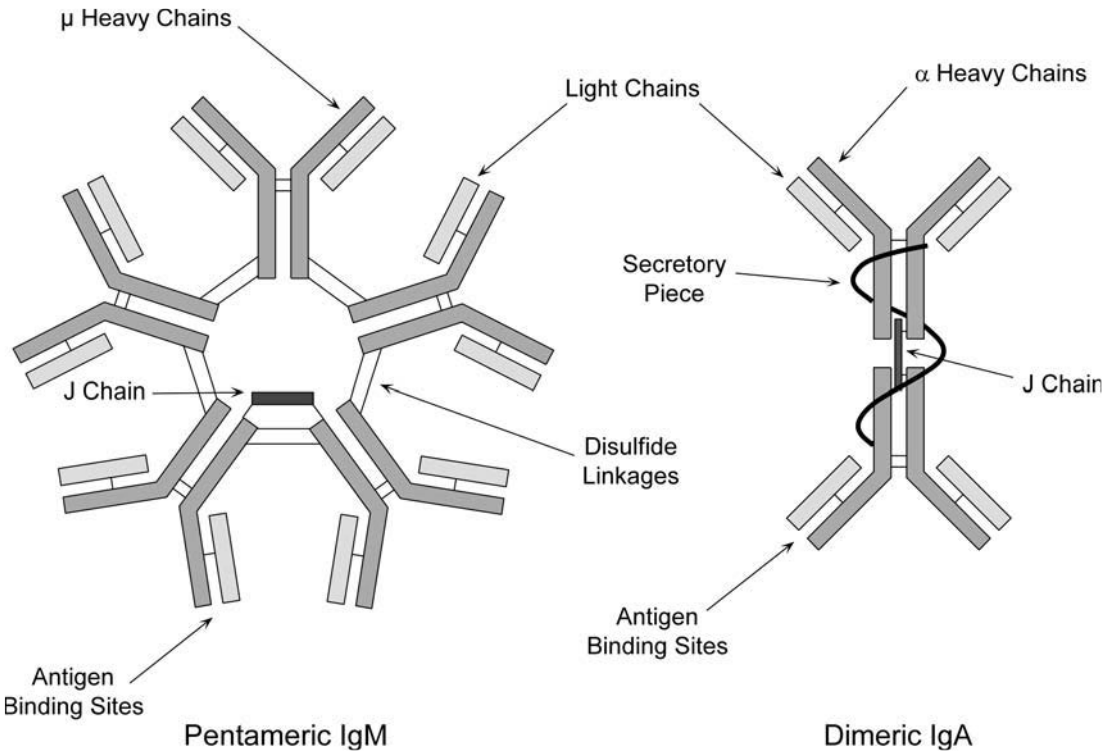

Figure 20.2 General structures of IgA and IgM antibodies.

the conformation necessary to interact with a complementary antigen molecule. The points of interaction on the immunoglobulin molecule with an antigen involve noncovalent forces that may encompass numerous nonsequential amino acids within the heavy and light chains. In other words, the binding site is formed not strictly from the linear sequence of amino acids on each chain, but from the unique orientation of these groups in three-dimensional space. The binding site thus has affinity for a particular antigen molecule due to both structural complementarity as well as the combination of van der Waals, ionic, hydrophobic, and hydrogen bonding forces which may be created at each point of contact.

Useful enzymatic derivatives of antibody molecules may be prepared that still retain the antigen binding sites. Two principal digested forms of IgG antibodies are useful for creating immunological reagents. Enzymatic digestion with papain produces two small fragments of the immunoglobulin molecule, each containing an antigen binding site (called Fab fragments), and one larger fragment containing only the lower portions of the two heavy chains (called Fc, for "fragment crystallizable") (Section 1.4, this chapter) (Coulter and Harris, 1983). Alternatively, pepsin cleavage produces one large fragment containing two antigen binding sites [called $F(ab')_2$] and many smaller fragments formed from extensive degradation of the Fc region (Rousseaux *et al.*, 1983). The $F(ab')_2$ fragment is held together by retention of the disulfide bonds in the hinge region. Specific reduction of these disulfides using 2-mercaptoethylamine (MEA) or other reducing agents (Chapter 1, Section 4.1) produces two Fab′ fragments, each of which has one antigen binding site.

Antibody molecules possess a number of functional groups suitable for modification or conjugation purposes. Crosslinking reagents may be used to target lysine ε-amine and N-terminal α-amine groups. Carboxylate groups also may be coupled to another molecule using the C-terminal end as well as aspartic acid and glutamic acid residues. Although both amine and carboxylate groups are as plentiful in antibodies as they are in most proteins, the distribution of them within the three-dimensional structure of an immunoglobulin is nearly uniform throughout the surface topology. For this reason, conjugation procedures that utilize these groups will crosslink somewhat randomly to nearly all parts of the antibody molecule. This in turn leads to a random orientation of the antibody within the conjugate structure, often blocking the antigen binding sites against the surface of another coupled protein or molecule. Obscuring the binding sites in this manner results in decreased antigen binding activity in the conjugate compared to that observed for the unconjugated antibody.

Conjugation chemistry done with antibody molecules generally is more successful at preserving activity if the functional groups utilized are present in limiting quantities and only at discrete sites on the molecule. Such "site-directed conjugation" schemes make use of crosslinking reagents that can specifically react with residues that are only in certain positions on the immunoglobulin surface—usually chosen to be well removed from the antigen binding sites. By proper selection of the conjugation chemistry and knowledge of antibody structure, the immunoglobulin molecule can be oriented so that its bivalent binding potential for antigen remains available.

Two site-directed chemical reactions are especially useful in this regard. The disulfides in the hinge region that hold the heavy chains together can be selectively cleaved with a reducing agent (such as MEA, DTT, or TCEP) to create two half-antibody molecules, each containing an antigen binding site (Palmer and Nissonoff, 1963; Sun *et al.*, 2005) (Chapter 1, Section 4.1). Alternatively, smaller antigen binding fragments may be made from pepsin digestion [$F(ab')_2$] and similarly reduced to form Fab′ molecules. Both of these preparations contain exposed

sulfhydryl groups which can be targeted for conjugation using thiol-reactive probes or crosslinkers. Conjugations done using hinge area —SH groups will orient the attached protein or other molecule away from the antigen binding regions, thus preventing blockage of these sites and preserving activity.

The second method of site-directed conjugation of antibody molecules takes advantage of the carbohydrate chains typically attached to the C_H2 domain within the Fc region. Mild oxidation of the polysaccharide sugar residues with sodium periodate will generate aldehyde groups. A crosslinking or modification reagent containing a hydrazide functional group then can be used to target specifically these aldehydes for coupling to another molecule. Directed conjugation through antibody carbohydrate chains thus avoids the antigen binding regions while allowing for use of intact antibody molecules. This method often results in the highest retention of antigen binding activity within the ensuing conjugate. However, care should be taken in using this method, because some antibody molecules can be glycosylated near the antigen binding area, thus potentially interfering with activity upon conjugate formation.

Another limitation to the use of this strategy is the necessity for the antibody molecule to be glycosylated. Antibodies of polyclonal origin (from antisera) are usually glycosylated and work well in this procedure, but other antibody preparations may not possess polysaccharide. In particular, some monoclonals may not be post-translationally modified with carbohydrate after hybridoma synthesis. Recombinant antibodies grown in bacteria also may be devoid of carbohydrate. Before attempting to use a conjugation method that couples through polysaccharide regions, it is best to test the antibody to see if it contains carbohydrate—especially if the immunoglobulin is of hybridoma or recombinant origin.

1. Preparation of Antibody–Enzyme Conjugates

The most extensive application of antibody conjugation using crosslinking reagents is for the preparation of antibody–enzyme conjugates. Since the development of enzyme-linked immunosorbent assay (ELISA) systems, the ability to make conjugates of specific antibodies with enzymes has provided the means to quantify or detect hundreds, if not thousands, of important analytes. The use of enzymes as labels in immunoassay procedures surpassed radioactive tags as the means of detection, primarily due to the long-term stability potential of an enzyme system and the hazards and waste problems associated with radioisotopes. Designed properly, an antibody–enzyme conjugate assay system can be just as sensitive as a radiolabeled antibody system.

The development of viable methods for crosslinking antibody and enzyme molecules—methods that retain high antigen binding activity coupled with high enzymatic activity—have formed the basis for much of today's diagnostic industries, literally a multi-billion dollar enterprise with enormous impact on world health. The conjugation chemistries that make this possible are designed around a knowledge of both antibody and enzyme structure. The best methods make use of definitive site-directed chemistries that target both molecules in regions removed from their respective active centers.

The major enzymes used in ELISA technology include horseradish peroxidase (HRP), alkaline phosphatase (AP), β-galactosidase (β-gal), and glucose oxidase (GO). See Chapter 26 for a detailed description of enzyme properties and activities. HRP is by far the most popular enzyme used in antibody–enzyme conjugates. One survey of enzyme use stated that HRP is incorporated in about 80 percent of all antibody conjugates, most of them utilized in diagnostic assay systems.

AP is the second most popular choice for antibody–enzyme conjugation, being used in almost 20 percent of all commercial enzyme-linked assays. Although β-gal and GO are used frequently in research and cited numerous times in the literature, their utilization for commercial ELISA applications represents less than 1 percent of the total assays available.

Conjugation methods for attaching these enzymes to antibody molecules vary according to the functional groups available. HRP is a glycoprotein and easily can be periodate oxidized for coupling via reductive amination to the amino groups on immunoglobulins. β-Gal contains abundant free sulfhydryl groups in its native state. The thiols can be utilized for coupling to the sulfhydryl-reactive end of heterobifunctional crosslinkers such as SMCC (Chapter 5, Section 1.3). Any of the enzymes can be conjugated through their amine groups using crosslinking agents such as glutaraldehyde or various heterobifunctional agents. The catalytic properties and activation methods often used with these enzymes are discussed in detail in Chapter 26.

The following sections describe the most common chemistries used to create antibody–enzyme conjugates.

1.1. NHS Ester–Maleimide-Mediated Conjugation

Heterobifunctional reagents containing an amine-reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on the other end generally have great utility for producing antibody–enzyme conjugates (see Chapter 5, Section 1). Crosslinking reagents possessing these reactive groups can be used in highly controlled, multi-step procedures that yield conjugates of defined composition and high activity. Among the most popular of these NHS ester–maleimide crosslinkers are SMCC (Chapter 5, Section 1.3), MBS (Chapter 5, Section 1.4), and GMBS (Chapter 5, Section 1.7). The use of any one of these crosslinkers in the following protocol can result in useful conjugates. However, SMCC and its water-soluble analog, sulfo-SMCC, possess the most stable maleimide functionalities and are probably the most widely used crosslinkers of this type. This increased stability to hydrolysis of SMCC's hindered maleimide group allows activation of either enzyme or antibody via the amine-reactive NHS ester end, resulting in a maleimide-activated intermediate. The intermediate species then can be purified away from excess crosslinker and reaction by-products before mixing with the second protein to be conjugated. The multi-step nature of this process limits polymerization of the conjugated proteins and provides control over the extent and sites of crosslinking.

In addition, the PEG-based heterobifunctional crosslinkers described in Chapter 18, Section 2, provide enhanced water-solubility for antibody conjugation applications. Conjugation of antibody molecules using a maleimide–PEG$_n$–NHS ester compound actually increases the solubility of the antibody and may help to maintain stability for certain sensitive monoclonals better than the traditional aliphatic crosslinkers. The methods described below for SMCC may be used with success for PEG-based reagents or other maleimide–NHS ester heterobifunctionals.

In protocols involving enzyme activation with SMCC and subsequent conjugation with an antibody molecule (the most common method of producing antibody–enzyme conjugates with this crosslinker), the antibody usually has to be prepared for coupling to the maleimide groups on the enzyme by introduction of sulfhydryl residues. Since antibodies typically do not contain free sulfhydryls accessible for conjugation, they must be fabricated by chemical means. Two main options are available for creating sulfhydryl functions on immunoglobulin molecules. The disulfide residues in the hinge region of the IgG structure may be reduced with DTT, TCEP, or

MEA to cleave the immunoglobulin into two half-antibody molecules each possessing one antigen binding site and the requisite sulfhydryls. Alternatively, a thiolation reagent may be used to modify the intact antibody to contain sulfhydryls (Chapter 1, Section 4.1). Both options are described below. Although there are numerous thiolation reagents from which to choose, only SATA and Traut's reagent are discussed in this section, since they are the most popular.

Activation of Enzymes with NHS Ester–Maleimide Crosslinkers

The first step in conjugation of antibody molecules and enzymes using NHS ester–maleimide crosslinkers usually is modification of the enzyme with the NHS ester end of the reagent to produce a maleimide-activated derivative (Figure 20.3). The protocol described here uses sulfo-SMCC as the crosslinking agent due to the enhanced stability of its maleimide group and the water-solubility afforded by the negatively charged sulfonate on its NHS ring. Other NHS ester–maleimide crosslinkers may be substituted without difficulty; however, water-insoluble varieties should be solubilized in DMSO or DMF prior to addition to the aqueous reaction mixture.

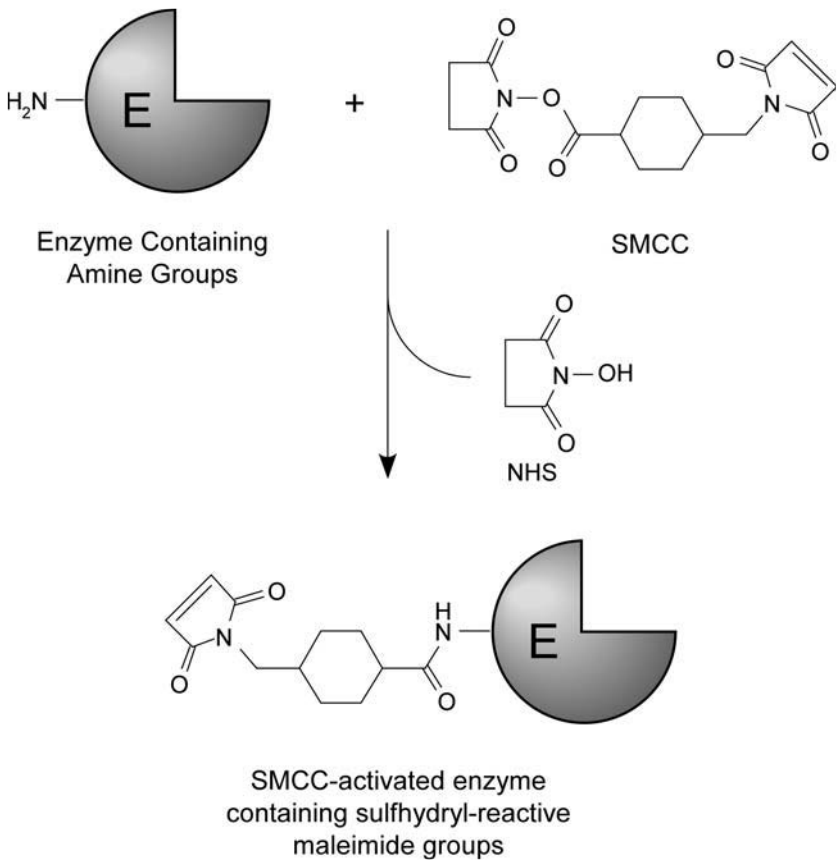

Figure 20.3 The reaction of SMCC with the amine groups on enzyme molecules yields a maleimide-activated derivative capable of coupling with sulfhydryl-containing antibody molecules.

One note should be mentioned before proceeding: when conjugating antibody molecules with β-galactosidase, the antibody usually is activated with sulfo-SMCC first to take advantage of the indigenous sulfhydryl groups on the enzyme. Therefore, if β-gal is being used, substitute the antibody for the enzyme mentioned in this protocol, and then after the purification step, add the enzyme in the desired molar excess to produce the final conjugation.

The following protocol describes the activation of HRP with sulfo-SMCC. Activation of other enzymes is done similarly, with the appropriate adjustments in the mass of enzyme added to the reaction to account for differences in molecular weight.

The gel filtration column described in step 3 should be prepared and equilibrated prior to starting the modification reaction. Enzymes preactivated with sulfo-SMCC are available from Thermo Fisher.

Protocol

1. Dissolve 18 mg of HRP in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 20–30 mg/ml. The more highly concentrated the enzyme solution, the more efficient will be the modification reaction. For conjugating smaller quantities of enzyme and antibody, proportionally decrease the amount of the reagents used, while attempting to maintain the same relative concentrations in solution.
2. Add 6 mg of sulfo-SMCC (Thermo Fisher) to the HRP solution. Mix to dissolve and react for 30 minutes at room temperature. Alternatively, two 3 mg additions of crosslinker may be done—the second one after 15 minutes of incubation—to obtain even more efficient modification.
3. Immediately purify the maleimide-activated HRP away from excess crosslinker and reaction by-products by gel filtration using a desalting resin. Use 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, as the chromatography buffer. At this concentration, HRP can be observed visually as it flows through the column due to the color of its heme ring. Pool the fractions containing the HRP peak. After elution, adjust the HRP concentration to 10 mg/ml for the conjugation reaction. At this point, the maleimide-activated enzyme may be frozen and lyophilized to preserve its maleimide activity. The modified enzyme is stable for at least 1 year in a freeze-dried state. If kept in solution, the maleimide-activated HRP should be used immediately to conjugate with an antibody following one of the three options outlined below.

Conjugation with Reduced Antibodies

One method of introducing sulfhydryl residues into antibody molecules for conjugation with maleimide-activated enzymes is to reduce indigenous disulfide groups in the hinge region of the immunoglobulin structure. Reduction with low concentrations of DTT, TCEP, or MEA will cleave principally the disulfide bonds holding the heavy chains together, but leave the disulfides between the heavy and light chains relatively intact. In a comparative study of disulfide reducing agents, it was determined that use of the relatively strong reductants DTT and TCEP required only 3.25 and 2.75 mole equivalents per mole equivalent of antibody molecule to achieve the reduction of two interchain disulfide bonds between the heavy chains of a monoclonal IgG (Sun *et al.*, 2005). This limited reduction strategy retains intact bispecific antibody molecules while providing discrete sites for conjugation to thiols. Using higher concentrations of DTT, TCEP, or MEA will result in complete cleavage of the disulfides between the heavy chains and formation of two half-antibody molecules, each containing an antigen binding site. Under these conditions,

some interchain cleavage also will occur and result in some smaller fragments being produced. Similar reduction can be done with $F(ab')_2$ fragments produced from pepsin digestion of IgG molecules. Either of these reduction steps creates half-antibody fragments, each containing one heavy and one light chain and one antigen binding site (Figure 20.4). The sulfhydryl groups

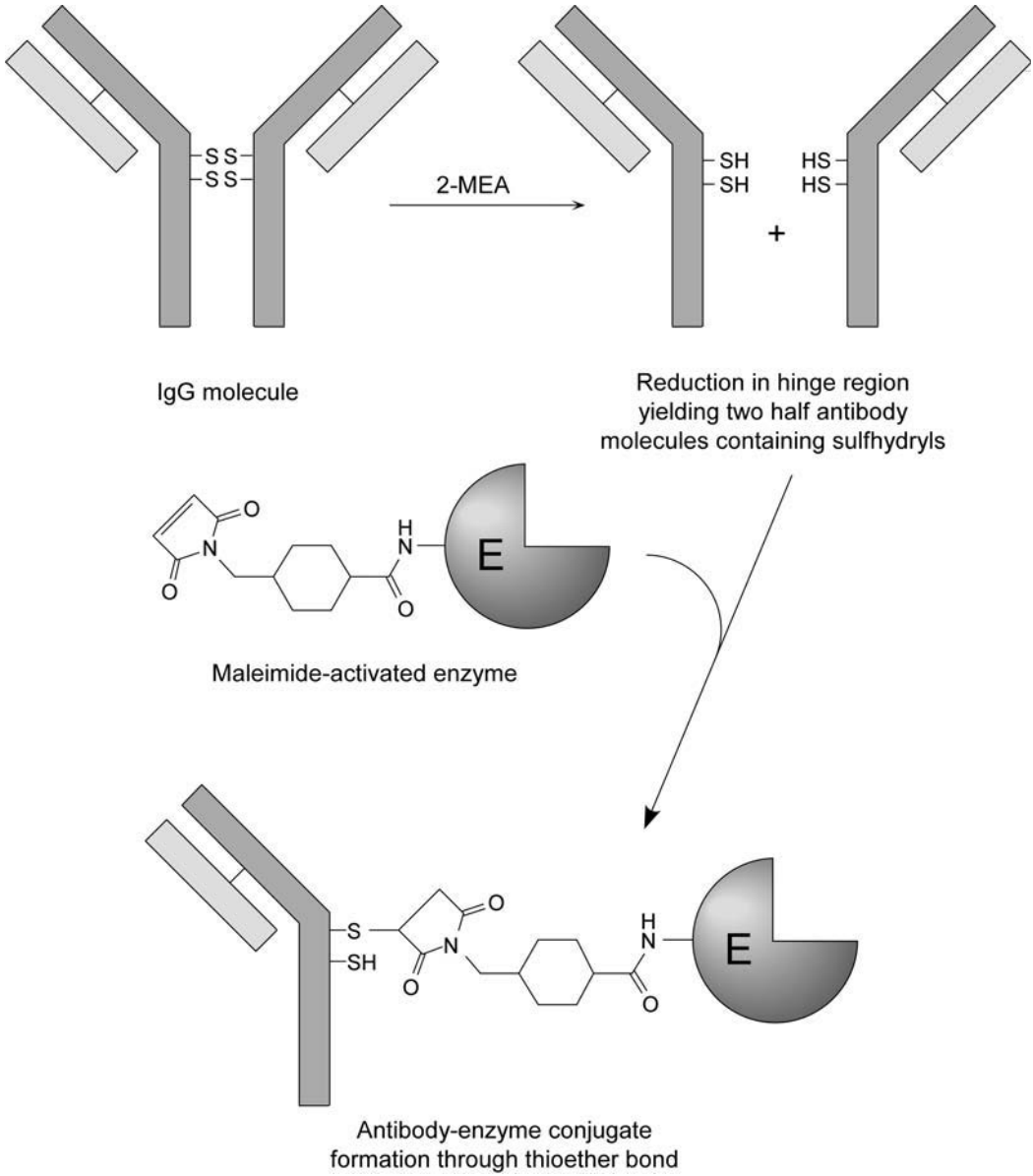

Figure 20.4 Reduction of the disulfide bonds within the hinge region of an IgG molecule produces half-antibody molecules containing thiol groups. Reaction of these reduced antibodies with a maleimide-activated enzyme creates a conjugate through thioether bond formation.

produced by this reduction are able to couple with maleimide-activated enzymes without blocking the antigen binding area.

Antibody reduction usually is done in the presence of EDTA to prevent re-oxidation of the sulfhydryls by metal catalysis. In phosphate buffer at pH 6–7 and 4°C, one report stated that the number of available thiols decreased only by about 7 percent in the presence of EDTA over a 40-hour time span. In the absence of EDTA, this sulfhydryl loss increased to 63–90 percent in the same period (Yoshitake *et al.*, 1979).

In the following protocol, the most critical aspects are the concentration of reducing agent and EDTA in the reaction mixture. Good reduction of IgG will take place with 50–100 mM MEA and 1–100 mM EDTA. For DTT or TCEP, the concentration of reducing agent should be lowered to a 3-fold molar excess over the amount of antibody present. The pH of the reaction can vary from pH 6 to 9, with about pH 8 being optimal. The absolute concentration of antibody can vary and still yield acceptable results. With some monoclonals, however, reduction may not be completely efficient in cleaving the antibody between the heavy chain pairs. Particularly, some subclasses of immunoglobulins contain structures with unusually high numbers of disulfides in the hinge region, and some of them may not be reduced except under much higher concentrations of reductant. Polyclonal populations typically work well in this procedure.

A final consideration is to provide adequate desalting of the reduced antibody molecule from excess reducing agent. If even a small amount of a thiol-containing reductant remains, subsequent conjugation with a maleimide-activated enzyme will be inhibited.

Protocol

1. Dissolve the IgG to be reduced at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA.
2. Add 6 mg of MEA to each ml of antibody solution. Alternatively, add DTT or TCEP to a final concentration equal to 3 mole equivalents per mole equivalent of antibody present. Mix to dissolve.
3. Incubate for 90 minutes at 37°C.
4. Purify the reduced IgG by gel filtration using a desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA as the buffer. To obtain efficient separation between the reduced antibody and excess reductant, the sample size applied to the column should be at a ratio of no more than 5 percent sample volume to column volume. Collect 0.5 ml fractions and monitor for protein at 280 nm. Since the reducing agents typically have no absorbance at 280 nm, the elution profile also may be monitored by use of the BCA Protein Assay method (Thermo Fisher). The BCA–copper reagent reacts with the reductants to produce a colored product. EDTA in the chromatography buffer will inhibit the BCA method somewhat, but a color response to the reducing agent peak will still be obtained. A micro-method for monitoring each fraction is as follows:

    a. Take 5 µl from each fraction collected and place in a separate well of a microtiter plate.
    b. Add 200 µl of BCA working reagent.
    c. Incubate at room temperature or 37°C for 15–30 minutes or until color develops. The color response may be measured visually or by absorbance at 562 nm. To assure good separation between the antibody peak and excess MEA, at least one fraction of little or no color should separate the two peaks.

5. Pool the fractions containing antibody and immediately mix with an amount of maleimide-activated enzyme to obtain the desired molar ratio of antibody-to-enzyme in the conjugate. Use of a 4:1 (enzyme:antibody) molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures. Higher molar ratios also have been used with success.
6. React for 30–60 minutes at 37°C or 2 hours at room temperature. The conjugation reaction also may be done at 4°C overnight.
7. The conjugate may be further purified away from unconjugated enzyme by the procedures described in Section 1.5, this chapter. For storage, the conjugate should be kept frozen, lyophilized, or sterile filtered and kept at 4°C. Stability studies may have to be done to determine the optimal method of long-term storage for a particular conjugate.

Conjugation with 2-Iminothiolane-Modified Antibodies

Traut's reagent, or 2-iminothiolane, is described in Chapter 1, Section 4.1. The reagent reacts with amine groups in proteins or other molecules in a ring-opening reaction to result in permanent modifications containing terminal sulfhydryl residues (Figure 20.5). Antibodies may be modified with Traut's reagent to create the requisite sulfhydryls necessary for conjugation with a maleimide-activated enzyme. Unlike the disulfide reduction method described in the previous section, this protocol better retains the divalent nature of the antibody molecule. However, since amine modification of antibodies can take place at virtually any available lysine ε-amine location, the resultant sulfhydryls are distributed almost randomly over the immunoglobulin structure. Conjugation through these —SH groups may result in a certain population of antibodies that have their antigen binding sites obscured or blocked by enzyme molecules. Typically, enough free antigen binding sites are available in the conjugate to result in high-activity complexes useful in ELISA procedures.

The number of sulfhydryls created on the immunoglobulin using thiolation procedures such as this one is more critical to the yield of conjugated enzyme molecules than the molar excess of maleimide-activated enzyme used in the conjugation reaction. Therefore, it is important to use a sufficient excess of Traut's reagent to obtain a sufficient number of available sulfhydryls.

Protocol

1. Dissolve the antibody to be modified at a concentration of 1–10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA. High levels of EDTA often are required to stop completely metal-catalyzed oxidation of sulfhydryl groups when working with serum proteins—especially polyclonal antibodies purified from antisera. Presumably, carry-over of iron from partially hemolyzed blood is the contaminating culprit.
2. Add 2-iminothiolane (Thermo Fisher) to this solution to give a molar excess of 20–40 × over the amount of antibody present (MW of Traut's reagent is 137.63). Addition of solid 2-iminothiolane may be done despite the fact that the compound is relatively insoluble in aqueous solution. As the reagent reacts, it will be completely drawn into solution. Alternatively, a stock solution of Traut's may be made in DMF and an aliquot added to the antibody solution (not to exceed 10 percent DMF in the final solution).
3. React for 30 minutes at 37°C or 1 hour at room temperature.

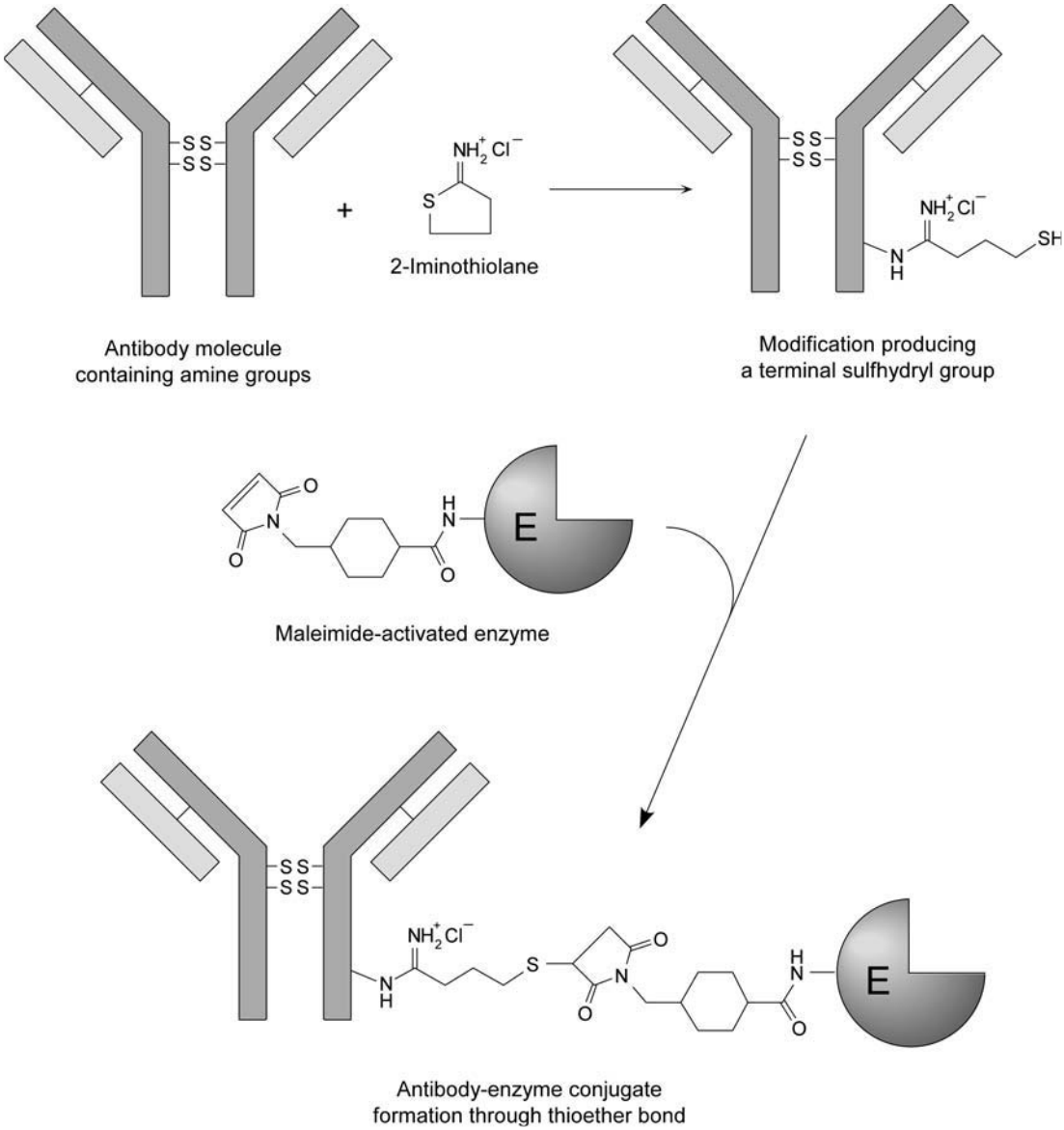

Figure 20.5 Antibodies may be modified with 2-iminothiolane at their amine groups to create sulfhydryls for conjugation with SMCC-activated enzymes. The maleimide groups on the derivatized enzyme react with the thiols on the antibody to form thioether bonds.

4. Purify the thiolated antibody by gel filtration using a desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA as the buffer. To obtain efficient separation between the reduced antibody and excess reductant, the sample size applied to the column should be at a ratio of no more

than 5 percent sample volume to the total column volume. Collect 0.5 ml fractions and monitor for protein at 280 nm. To monitor the separation of the second peak (excess Traut's reagent), the BCA Protein Assay reagent (Thermo Fisher) may be used according to the procedure described in the previous section, protocol step 4.

5. Pool the fractions containing antibody and immediately mix with an amount of maleimide-activated enzyme to obtain the desired molar ratio of antibody-to-enzyme in the conjugate. Use of a 4:1 (enzyme:antibody) to 15:1 molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures.
6. React for 30–60 minutes at 37°C or 2 hours at room temperature. The conjugation reaction also may be done at 4°C overnight.
7. The conjugate may be further purified away from unconjugated enzyme by the procedures described in Section 1.5, this chapter. For storage, the conjugate should be kept frozen, lyophilized, or sterile filtered and kept at 4°C. Stability studies may have to be done to determine the optimal method of long-term storage for a particular conjugate.

Conjugation with SATA-Modified Antibodies

N-Succinimidyl-*S*-acetylthioacetate (SATA) is a thiolation reagent described in detail in Chapter 1, Section 4.1. The compound reacts with primary amines via its NHS ester end to form stable amide linkages. The acetylated sulfhydryl group is stable until deacetylated with hydroxylamine. Thus, antibody molecules may be thiolated with SATA to create the sulfhydryl target groups necessary to couple with a maleimide-activated enzyme (Figure 20.6). Using this reagent, stock preparations of SATA-modified antibodies may be prepared and deacetylated as needed. Unlike thiolation procedures which immediately form a free sulfhydryl residue, the protected sulfhydryl group of SATA-modified proteins is stable to long-term storage without degradation.

Although amine-reactive protocols, such as SATA thiolation, result in nearly random attachment over the surface of the antibody structure, it has been shown that modification with up to 6 SATAs per antibody molecule typically results in no decrease in antigen binding activity (Duncan *et al*., 1983). Even higher ratios of SATA to antibody are possible with excellent retention of activity.

The following protocol should be compared to the method described for SATA thiolation in Chapter 1, Section 4.1. Although the procedures are slightly dissimilar, the differences indicate the flexibility inherent in the chemistry. For convenience, the buffer composition indicated here was chosen to be consistent throughout this section on enzyme–antibody conjugation using SMCC. Other buffers and alternate protocols can be found in the literature.

Protocol

1. Dissolve the antibody to be modified in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 1–5 mg/ml. *Note*: Phosphate buffers at various pH values between 7.0 and 7.6 have been used successfully with this protocol. Other mildly alkaline buffers may be substituted for phosphate in this reaction, providing they don't contain extraneous amines (e.g., Tris) or promote hydrolysis of SATA's NHS ester (e.g., imidazole).
2. Prepare a stock solution of SATA (Thermo Fisher) by dissolving it in DMF or DMSO at a concentration of 8 mg/ml. Use a fume hood to handle the organic solvents.

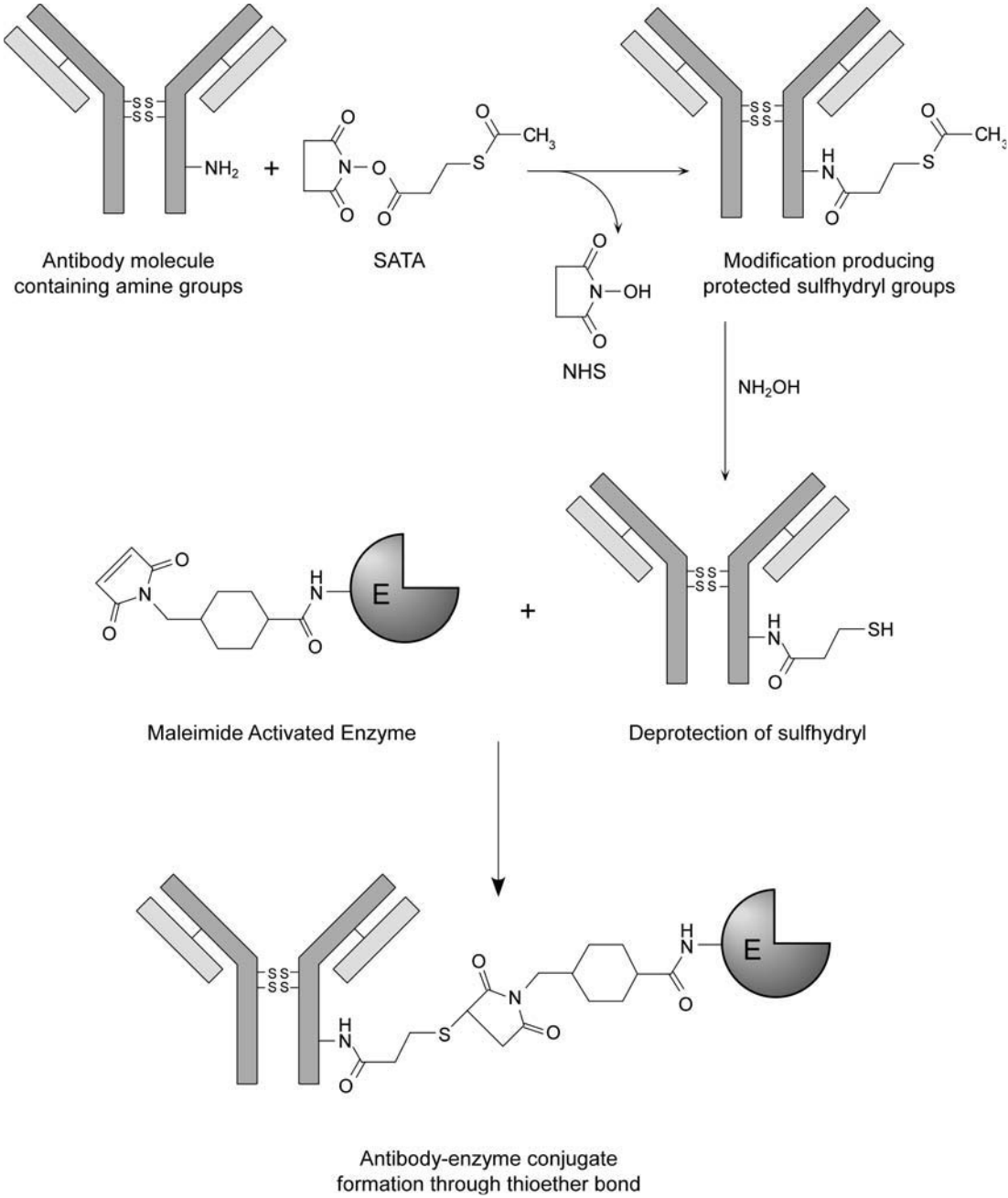

Figure 20.6 Available amine groups on an antibody molecule may be modified with the NHS ester end of SATA to produce amide bond derivatives containing terminal protected sulfhydryls. The acetylated thiols may be deprotected by treatment with hydroxylamine at alkaline pH. Reaction of the thiolated antibody with a maleimide-activated enzyme results in thioether crosslinks.

3. Add 10–40 µl of the SATA stock solution per ml of 1 mg/ml antibody solution. This will result in a molar excess of approximately 12- to 50-fold of SATA over the antibody concentration (for an initial antibody concentration of 1 mg/ml). A 12-fold molar excess works well, but higher levels of SATA incorporation will potentially result in more maleimide-activated enzyme molecules able to couple to each thiolated antibody molecule. For higher concentrations of antibody in the reaction medium, proportionally increase the amount of SATA addition; however do not exceed 10 percent DMF in the aqueous reaction medium.
4. React for 30 minutes at room temperature.
5. To purify the SATA-modified antibody perform a gel filtration separation using desalting resin or by dialysis against 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA. Purification is not absolutely required, since the following deprotection step is done using hydroxylamine at a significant molar excess over the initial amount of SATA added. Whether a purification step is done or not, at this point, the derivative is stable and may be stored under conditions which favor long-term antibody activity (i.e., sterile filtered at 4°C, frozen, or lyophilized).
6. Deprotect the acetylated sulfhydryl groups on the SATA-modified antibody according to the following protocol:
   a. Prepare a 0.5 M hydroxylamine (Thermo Fisher) solution in 0.1 M sodium phosphate, pH 7.2, containing 10 mM EDTA.
   b. Add 100 µl of the hydroxylamine stock solution to each ml of the SATA-modified antibody. Final concentration of hydroxylamine in the antibody solution is 50 mM.
   c. React for 2 hours at room temperature.
   d. Purify the thiolated antibody by gel filtration on a desalting resin using 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.2, containing 10 mM EDTA as the chromatography buffer. To obtain efficient separation between the thiolated antibody and excess hydroxylamine and reaction by-products, the sample size applied to the column should be at a ratio of no more than 5 percent sample volume to the total column volume. Collect 0.5 ml fractions. Pool the fractions containing protein by measuring the absorbance of each fraction at 280 nm.
7. Immediately mix the thiolated antibody with an amount of maleimide-activated enzyme to obtain the desired molar ratio of antibody-to-enzyme in the conjugate. Use of a 4:1 (enzyme:antibody) to 15:1 molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures.
8. React for 30–60 minutes at 37°C or 2 hours at room temperature. The conjugation reaction also may be done at 4°C overnight.
9. The conjugate may be further purified away from unconjugated enzyme by the procedures described in Section 1.5, this chapter. For storage, the conjugate should be kept frozen, lyophilized, or sterile filtered and kept at 4°C. Stability studies may have to be done to determine the optimal method of long-term storage for a particular conjugate.

1.2. Glutaraldehyde-Mediated Conjugation

Glutaraldehyde was one of the first and still is one of the most commonly used crosslinking agents available for creating antibody–enzyme conjugates. The crosslinking process using

glutaraldehyde is believed to proceed by a number of mechanisms, including Schiff base formation with possible rearrangement to a stable product or through a Michael-type addition reaction that takes place at points of double-bond unsaturation created by polymerization of the reagent in solution (Chapter 1, Section 4.4, and Chapter 4, Section 6.2) (Avrameas, 1969). Reduction of Schiff base intermediates also is possible using sodium cyanoborohydride to form stable secondary amine linkages.

The problem of indeterminate reaction products is a deficiency that plagues all conjugations done using glutaraldehyde. Part of this difficulty is due to the reagent's homobifunctional nature, but a significant part of the problem is also due to the ambiguous nature of the commercial product. In aqueous solutions at alkaline pH, glutaraldehyde can undergo aldol condensation reactions with itself to form large polymer structures containing α,β-unsaturated aldehydes (Hardy *et al.*, 1969, 1976). Another disadvantage of the reagent is the tendency to form high-molecular-weight conjugates due to uncontrollable polymerization during the crosslinking process. The resultant conjugates often have a significant amount of insoluble polymer which causes yield and activity losses in the preparation of antibody–enzyme conjugates. This is especially true when the conjugation is done using the one-step method where glutaraldehyde is simply added to a solution containing the two proteins to be crosslinked (Figure 20.7). Enzymatic activity yields using this process can be as little as 10 percent in the final antibody–enzyme conjugate. To somewhat overcome the polymerization problem, a two-step procedure was developed which involves first activating one of the proteins with glutaraldehyde, purifying the intermediate from excess reagent, and then adding the second protein to effect the final conjugation. Unfortunately, even the two-step method results in significant formation of large molecular weight species that may precipitate out of solution. The only enzyme that the two-step method seems to work well with is HRP, since it only contains a limited number of available lysine amine groups.

Despite these deficiencies, antibody–enzyme conjugates are still being made using glutaraldehyde—particularly for many commercial diagnostic ELISA kits which were developed before the advent of more controllable, heterobifunctional crosslinking procedures. Today, choosing another method of producing antibody–enzyme conjugates will result in much better conjugates of higher activity and higher yield.

One-Step Glutaraldehyde Protocol

1. Prepare a solution containing 2 mg/ml antibody and 5 mg/ml enzyme in 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, chilled to 4°C.
2. In a fume hood, add 10 µl of 25 percent glutaraldehyde (Sigma) per ml of antibody/enzyme solution. Mix well.
3. React for 2 hours at 4°C.
4. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride (Aldrich) to a final concentration of 10 mg/ml.

   Note: Some protocols do not call for a reduction step. As an alternative to reduction, add 50 µl of 0.2 M lysine in 0.5 M sodium carbonate, pH 9.5 to each ml of the conjugation reaction to block excess reactive sites. Block for 2 hours at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.

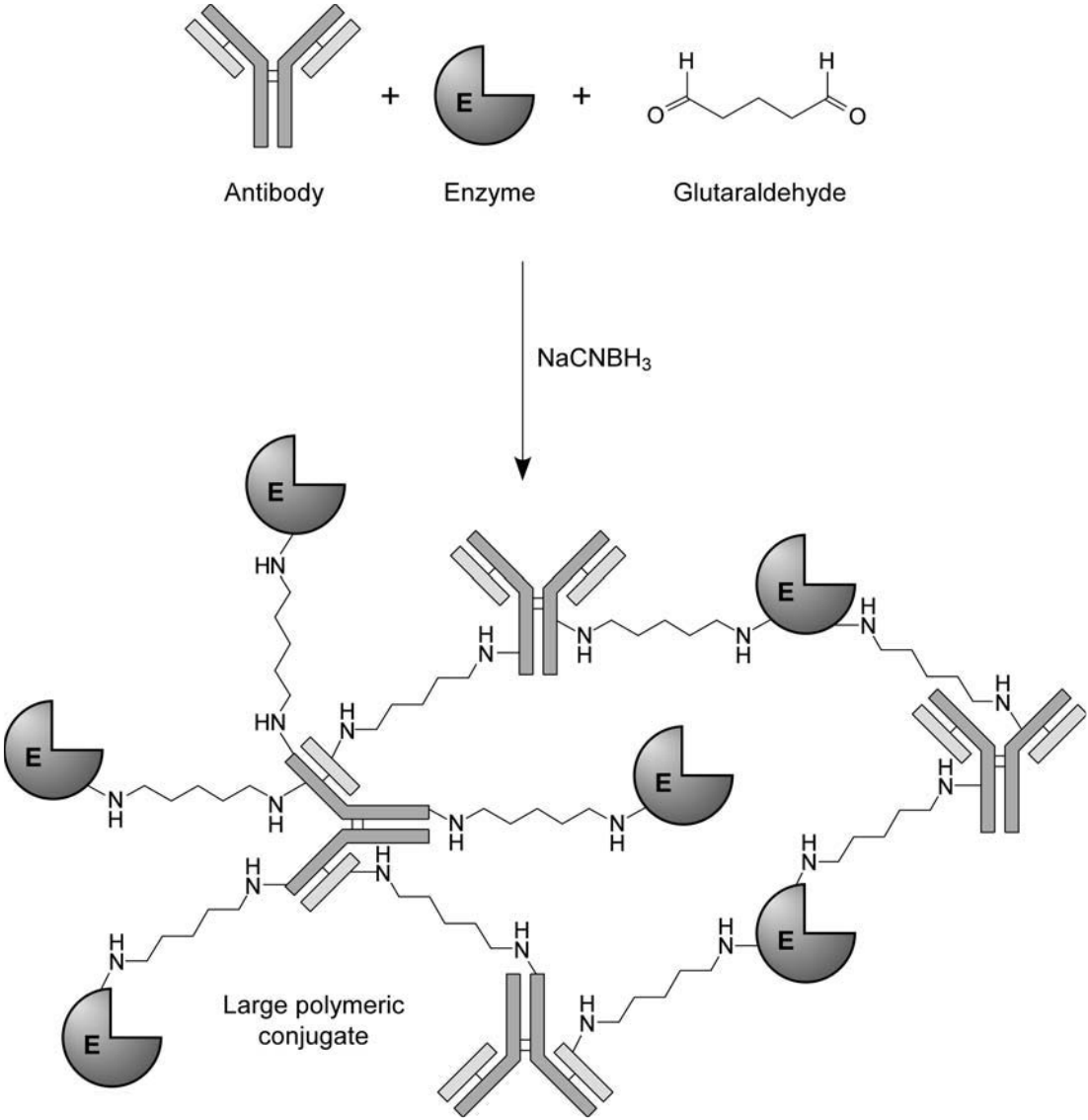

Figure 20.7 Glutaraldehyde antibody–enzyme crosslinking procedures usually produce a wide range of high-molecular-weight complexes, some of which may precipitate from solution.

5. Reduce for 1 hour at 4°C.
6. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45 µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

Two-Step Glutaraldehyde Protocol

1. Dissolve the enzyme at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8.
2. Add glutaraldehyde to a final concentration of 1.25 percent.
3. React overnight at room temperature.
4. Purify the activated enzyme from excess glutaraldehyde by gel filtration using a desalting resin or by dialysis against PBS, pH 6.8.
5. Dissolve the antibody to be conjugated at a concentration of 10 mg/ml in 0.5 M sodium carbonate, pH 9.5. Mix the activated enzyme with the antibody at the desired molar ratio to effect the conjugation. Mixing the equivalent of 4 mg of enzyme per mg of antibody usually results in acceptable conjugates.
6. React overnight at 4°C.
7. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.

   Note: Some protocols avoid a reduction step. As an alternative to reduction, add 50 µl of 0.2 M lysine in 0.5 M sodium carbonate, pH 9.5 to each ml of the conjugation reaction to block excess reactive sites. Block for 2 hours at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.
8. Reduce for 1 hour at 4°C.
9. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45 µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

1.3. Reductive Amination-Mediated Conjugation

Oxidation of polysaccharide residues in glycoproteins with sodium periodate provides an efficient way of generating reactive aldehyde groups for subsequent conjugation with amine- or hydrazide-containing molecules via reductive amination (Chapter 1, Section 4.4, and Chapter 2, Section 5.3). Some selectivity of monosaccharide oxidation may be accomplished by regulating the concentration of periodate in the reaction medium. In the presence of 1 mM sodium periodate at approximately 0°C sialic acid groups will be specifically oxidized at their adjacent hydroxyl residues on the Nos. 7, 8, and 9 carbon atoms, cleaving off two molecules of formaldehyde and leaving one aldehyde group on the No. 7 carbon. At higher concentrations of sodium periodate (10 mM or greater) at room temperature other sugar residues will be oxidized at points where adjacent carbon atoms contain hydroxyl groups.

Thus, glycoproteins such as HRP, GO, or most antibody molecules can be activated for conjugation by brief treatment with periodate. Crosslinking with an amine-containing protein takes place under alkaline pH conditions through the formation of Schiff base intermediates. These relatively labile intermediates can be stabilized by reduction to a secondary amine linkage with sodium cyanoborohydride (Figure 20.8).

The use of periodate coupling chemistry for HRP first was introduced by Nakane and Kawaoi (1974; see also Nakane, 1975). In the first step of their protocol, the few amine groups on HRP were initially blocked with 2,4-dinitrofluorobenzene (DNFB) before periodate oxidation.

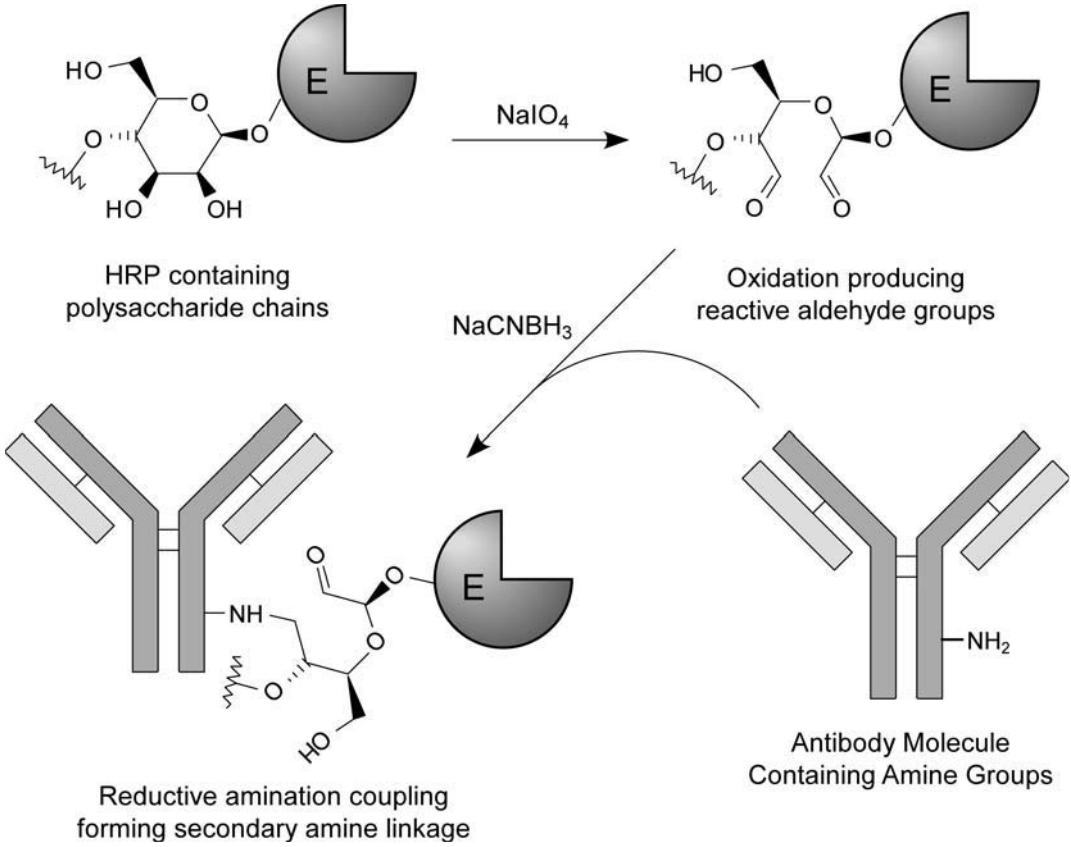

Figure 20.8 Enzymes that are glycoproteins like HRP may be oxidized with sodium periodate to produce reactive aldehyde residues. Conjugation with an antibody then may be done by reductive animation using sodium cyanoborohydride.

The blocking step was designed to eliminate the possibility of self-conjugation of enzyme molecules during reductive amination with an immunoglobulin. However, Boorsma and Streefkerk (1976a, b) determined that HRP still can dimerize even after DNFB blocking, perhaps by a mechanism similar to Mannich condensation (Chapter 2, Section 6.2) or through aldol formation. In fact, amine-blocked, periodate-oxidized HRP will form insoluble complexes during storage after just weeks in solution at room temperature or 4°C, indicating that another route of conjugation is taking place.

Reductive amination crosslinking has been done using sodium borohydride or sodium cyanoborohydride; however cyanoborohydride is the better choice since it is more specific for reducing Schiff bases and will not reduce aldehydes. Small blocking agents such as lysine, glycine, ethanolamine, or Tris can be added after conjugation to quench any unreacted aldehyde sites (Mannik and Downey, 1973; Barbour, 1976; Mattiasson and Nilsson, 1977). Ethanolamine and Tris are the best choices for blocking agents, since they contain hydrophilic hydroxyl groups with no charged functional groups.

The pH of the reductive amination reaction can be controlled to affect the efficiency of the crosslinking process and the size of the resultant antibody–enzyme complexes formed. At physiological pH, the initial Schiff base formation is less efficient and conjugates of lower molecular weight will result. At more alkaline pH values (i.e., pH 9–10), Schiff base formation occurs rapidly and with high efficiency, resulting in conjugates of higher molecular weight and greater incorporation of enzyme (when oxidized HRP is reacted in excess).

The ability to select the relative size of the antibody–enzyme complex is important depending on the assay application. Low-molecular-weight conjugates may be more optimal for immunohistochemical staining or blotting techniques where penetration of the complex through membrane barriers is an important consideration. Washing steps also more effectively remove excess reagent if the conjugate is of low molecular weight, thus maintaining a low background signal in an assay. By contrast, conjugates of high molecular weight are more appropriate for ELISA procedures in a microplate or array format, where high sensitivity is important, but washing off excess conjugate is not a problem.

The protocols appearing in the literature vary according to the amount of periodate used during polysaccharide oxidation, the type of reductant and blocking agent employed for reductive amination, and the pH at which the various reactions are done. This variability indicates considerable flexibility in the protocols, all of which yield usable antibody–enzyme conjugates. There are, however, several conclusions that can be drawn from these studies: Investigations done using HRP indicate the optimal concentration of sodium periodate during oxidation to be approximately 4–8 mM (Tussen and Kurstak, 1984). This reaction should be performed in the dark to prevent periodate breakdown and for a limited period of time (no more than 15–30 minutes) to avoid loss of enzymatic activity. The conjugation reaction should be done at alkaline pH (7.2–9.5) in the presence of a reducing agent to stabilize the Schiff base intermediates. If sodium cyanoborohydride is used as the reductant, a blocking agent should be added at the completion of the conjugation reaction to cap excess aldehyde sites. The following protocol follows these general guidelines and works well especially in the preparation of HRP–antibody conjugates.

Activation of Enzymes with Sodium Periodate

Enzymes that are glycosylated (i.e., HRP and GO) may be oxidized according to the following method to produce aldehyde groups for reductive amination coupling to an antibody molecule.

Protocol

1. Dissolve the enzyme to be oxidized in water or 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10–20 mg/ml.
2. Dissolve sodium periodate in water at a concentration of 0.088 M. Protect from light.
3. Immediately add 100 µl of the sodium periodate solution to each ml of the enzyme solution. This ratio of addition results in an 8 mM periodate concentration in the reaction mixture. Mix to dissolve. Protect from light.
4. React in the dark for 15–20 minutes at room temperature. If HRP is the enzyme being oxidized, a color change will be apparent as the reaction proceeds—changing the brownish/gold color of concentrated HRP to green. Limiting the time of oxidation will help to preserve enzyme activity.

5. Immediately quench the reaction by the addition of 0.1 ml of glycerol per ml of reaction solution. Instead of glycerol, *N*-acetylmethionine may be added to quench the reaction, because the thioether of the methionine side chain will react with periodate to form sulfoxide or sulfone products (Geoghegan and Stroh, 1992). In addition, sodium sulfite (Na_2SO_3) was used by Stolowitz *et al.* (2001) to quench the periodate oxidation of HRP in solution. This may be the simplest route to stopping the reaction, as sulfite is inexpensive and the reduction doesn't form reactive by-products. Add quenching reagent to provide at least a 2 × molar excess over the amount of periodate initially added to the reaction. Alternatively, the reaction may be stopped by immediate gel filtration on a desalting resin. If a dextran-based resin is used for the chromatography, the support itself will react with sodium periodate to quench excess reagent. Purify the oxidized enzyme by gel filtration using 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2. To obtain efficient separation between the oxidized enzyme and excess periodate (or quenching agent), the sample size applied to the column should be at a ratio of no more than 5 percent sample volume to the total column volume. Collect 0.5 ml fractions and monitor for protein at 280 nm. HRP also may be detected by its absorbance at 403 nm. When oxidizing large quantities of HRP, the fraction collection process may be done visually—just pooling the main colored HRP peak as it comes off the column.
6. Pool the fractions containing protein. Adjust the enzyme concentration to 10 mg/ml for the conjugation step (see next section). The periodate-activated enzyme may be stored frozen or freeze-dried for extended periods without loss of activity. Do not store the preparation in solution at room temperature or 4°C, since precipitation will occur over time due to self-polymerization.

Activation of Antibodies with Sodium Periodate

Many immunoglobulin molecules are glycoproteins that can be periodate-oxidized to contain reactive aldehyde residues. Polyclonal IgG molecules often contain carbohydrate in the Fc portion of the molecule. This is sufficiently removed from the antigen binding sites to allow conjugation to take place through the polysaccharide chains without compromising activity. Occasionally, however, some antibodies may contain sites of glycosylation near the antigen binding regions, and in this situation conjugation through these sites may affect binding activity. Although antibody–enzyme conjugation by reductive amination typically is done by oxidation of the enzyme with subsequent crosslinking to an amine-containing antibody, oxidation of the antibody with subsequent conjugation to an amine- or hydrazide-containing molecule also is possible. It should be noted, however, that many monoclonal antibodies are not glycosylated and therefore cannot be used in this protocol. Recombinant antibodies also do not contain carbohydrate. A given monoclonal should be checked to verify the presence of carbohydrate before attempting to use a periodate-mediated conjugation protocol.

Protocol

1. Dissolve the antibody to be periodate-oxidized at a concentration of 10 mg/ml in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. Dissolve sodium periodate in water to a final concentration of 0.1 M. Protect from light.

3. Immediately add 100 µl of the sodium periodate solution to each ml of the antibody solution. Mix to dissolve. Protect from light.
4. React in the dark for 15–20 minutes at room temperature.
5. Immediately quench the reaction by the addition of sodium sulfite (Na_2SO_3) to provide a 2× molar excess over the initial amount of periodate added. Purify the oxidized antibody by gel filtration using a desalting resin. The chromatography buffer is 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2. To obtain efficient separation between the oxidized antibody and excess periodate, the sample size applied to the column should be at a ratio of no more than 5 percent sample volume to the total column volume. Collect 0.5 ml fractions and monitor for protein at 280 nm.
6. Pool the fractions containing protein. Adjust the antibody concentration to 10 mg/ml for the conjugation step. The oxidized antibody should be used immediately.

Conjugation of Periodate-Oxidized HRP to Antibodies by Reductive Amination

The following protocol assumes that HRP has already been periodate-oxidized by the method of Section 1.3.

Protocol

1. Dissolve the IgG to be conjugated at a concentration of 10 mg/ml in 0.2 M sodium bicarbonate, pH 9.6, at room temperature. The high-pH buffer will result in very efficient conjugation with the highest possible incorporation of enzyme molecules per antibody molecule. To produce lower-molecular-weight conjugates, dissolve the IgG at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. The periodate-oxidized enzyme (HRP) prepared in Section 1.3 was finally purified using 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2. For conjugation using the lower-pH buffered environment, this HRP preparation can be used directly at 10 mg/ml concentration. For conjugation using the higher-pH carbonate buffer, dialyze the HRP solution against 0.2 M sodium carbonate, pH 9.6 for 2 hours at room temperature prior to use. A volume of HRP solution equal to the volume of antibody solution will be required.
3. Mix the antibody solution with the enzyme solution at a ratio of 1:1 (v/v). Since an equal mass of antibody and enzyme is present in the final solution, this will result in a 3.75 molar excess of HRP over the amount of IgG. For conjugates consisting of greater enzyme-to-antibody ratios, proportionally increase the amount of enzyme solution as required. Typically, molar ratios of 4:1 to 15:1 (enzyme:antibody) give acceptable conjugates useful in a variety of ELISA techniques.
4. React for 2 hours at room temperature.
5. In a fume hood, add 10 µl of 5 M sodium cyanoborohydride (Sigma) per ml of reaction solution. *Caution*: Cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 M solution is prepared in 1 N NaOH.
6. React for 30 minutes at room temperature with gentle mixing (in a fume hood).
7. Block unreacted aldehyde sites by addition of 50 µl of 1 M ethanolamine, pH 9.6, per ml of conjugation solution. Approximately a 1 M ethanolamine solution may be prepared

by addition of 300 µl ethanolamine to 5 ml of deionized water. Adjust the pH of the ethanolamine solution by addition of concentrated HCl, keeping the solution cool on ice.
8. React for 30 minutes at room temperature.
9. Purify the conjugate from excess reactants by dialysis or gel filtration using a desalting resin. Use 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0, as the buffer for either operation. The conjugate may be further purified by removal of unconjugated enzyme using one of the methods described in Section 1.5, this chapter.

Conjugation of Periodate-Oxidized Antibodies with Amine or Hydrazide Derivatives

The following protocol assumes that the antibody has already been periodate-oxidized by the method of Section 1.3 to create reactive aldehyde groups suitable for coupling with amine- or hydrazide-containing molecules. This is an excellent method for directing the antibody modification reaction away from the antigen binding sites, if the antibody glycosylation points are solely in the Fc region of the molecule. For instance, biotinylation of intact antibodies can be done after mild periodate treatment using biotin-hydrazide (Chapter 11, Section 3) (Figure 20.9). It should be noted, however, that periodate-oxidized antibodies can self-conjugate through their own amines if high-pH reductive amination is used. Conjugation with periodate-oxidized antibodies works best if the receiving molecule is modified to contain hydrazide groups and the reaction is done at more moderate pH values (e.g., slightly acidic to neutral pH).

1. For conjugation to hydrazide-containing proteins, dissolve the periodate-oxidized antibody at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.0–7.2. For conjugation to amine-containing molecules and proteins, dissolve the oxidized antibody at 10 mg/ml in 0.2 M sodium carbonate, pH 9.6.
2. Dissolve a hydrazide-containing enzyme or other protein at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.0–7.2. For the preparation of a hydrazide-activated enzyme, see Chapter 26, Section 2.4. For modification with a hydrazide-containing probe, such as biotin-hydrazide, use a concentration of 5 mM in the phosphate buffer. For conjugation through the amine groups of a secondary molecule, dissolve the amine-containing protein at 10 mg/ml in 0.2 M sodium carbonate, pH 9.6.
3. Mix the antibody solution from step 1 with the protein solution from step 2 in amounts necessary to obtain the desired molar ratio for conjugation. Often, the secondary molecule is reacted in approximately a 4- to 15-fold molar excess over the amount of antibody present.
4. React for 2 hours at room temperature.
5. In a fume hood, add 10 µl of 5 M sodium cyanoborohydride (Sigma) per ml of reaction solution. *Caution*: Cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 M solution is prepared in 1 N NaOH. The addition of a reductant is necessary for stabilization of the Schiff bases formed between an amine-containing protein and the aldehydes on the antibody. For coupling to a hydrazide-activated protein, however, most protocols do not include a reduction step. Even so, hydrazone linkages may be further stabilized by cyanoborohydride reduction. The addition of a reductant during hydrazide/aldehyde reactions also increases the efficiency and yield of the reaction.
6. React for 30 minutes at room temperature (in a fume hood).

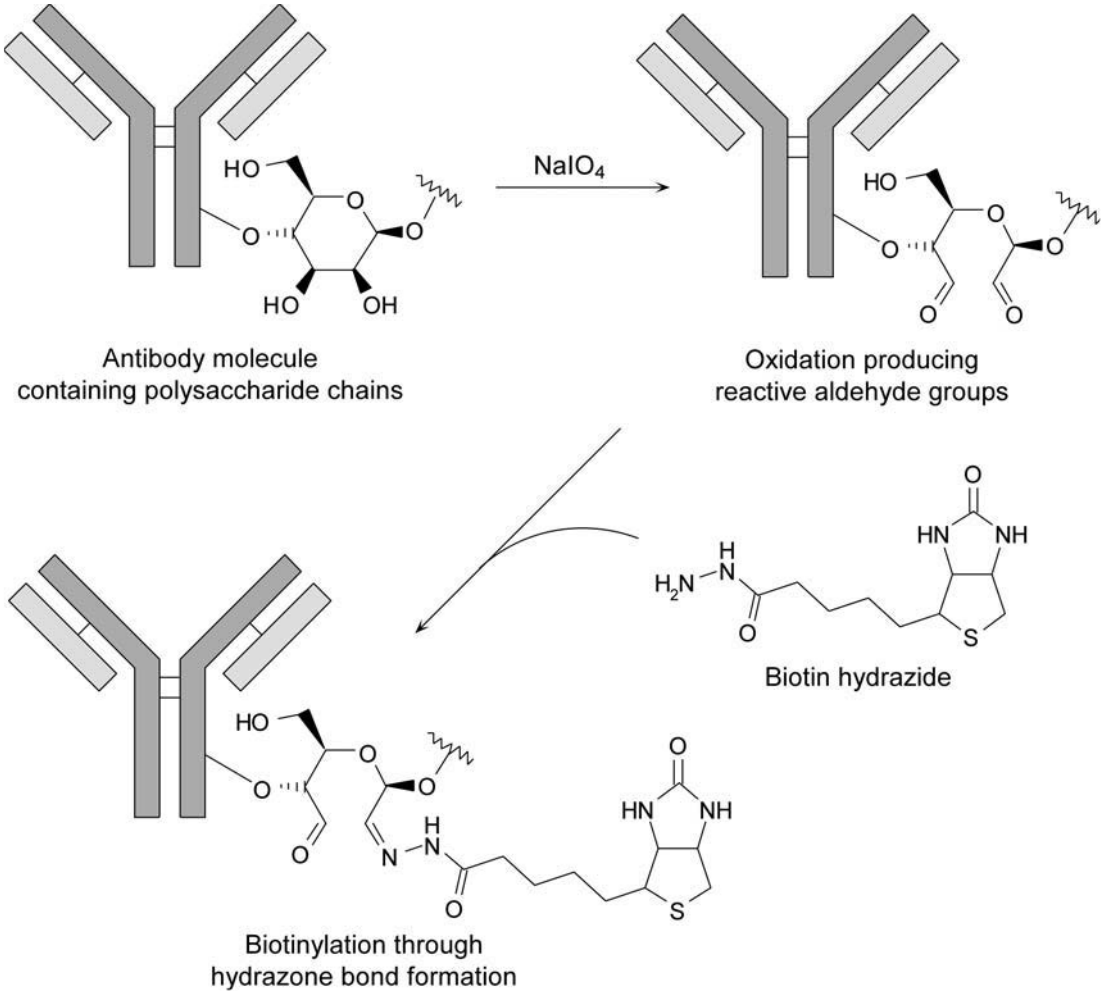

Figure 20.9 Polysaccharide groups on antibody molecules may be oxidized with periodate to create aldehydes. Modification with biotin-hydrazide results in hydrazone linkages. The sites of modification using this technique often are away from the antibody–antigen binding regions, thus preserving antibody activity.

7. Block unreacted aldehyde sites by addition of 50 µl of 1 M ethanolamine, pH 9.6, per ml of conjugation solution. Approximately a 1 M ethanolamine solution may be prepared by addition of 300 µl ethanolamine to 5 ml of deionized water. Adjust the pH of the ethanolamine solution by addition of concentrated HCl, while keeping the solution cool on ice.
8. React for 30 minutes at room temperature.
9. Purify the conjugate from excess reactants by dialysis or gel filtration using desalting resin. Use 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0, as the buffer for either operation. The conjugate may be further purified by removal of unconjugated enzyme by one of the methods of Section 1.5, this chapter.

1.4. Conjugation Using Antibody Fragments

It is often advantageous to use antibody fragments in the preparation of antibody–enzyme conjugates. Selected fragmentation carried out by enzymatic digestion of intact immunoglobulins can yield lower-molecular-weight molecules still able to recognize and bind antigen. Conjugation of these fragments with enzyme molecules can result in ELISA reagents that possess better characteristics than corresponding conjugates prepared with intact antibody. Such antibody fragment conjugates display less interference with various Fc binding proteins and also less immunogenicity (due to lack of the Fc region), more facile membrane penetration for immunohistochemical staining techniques (due to lower overall conjugate molecular weight) (Wilson and Nakane, 1978; Farr and Nakane, 1981), and lower nonspecific binding to surfaces or membranes (resulting in increased signal-to-noise ratios) (Hamaguchi *et al*., 1979; Ishikawa *et al*., 1981a, b).

Enzymatic digests of IgG can result in two particularly useful fragments called Fab and $F(ab')_2$, prepared by the action of papain and pepsin, respectively. Most specific enzymatic cleavages of IgG occur in relatively unfolded regions between the major domains. Papain and pepsin, and similar enzymes including bromelain, ficin, and trypsin, cleave immunoglobulin molecules in the hinge region of the heavy chain pairs. Depending on the location of cleavage, the disulfide groups holding the heavy chains together may or may not remain attached to the antigen binding fragments that result. If the disulfide-bonded region does remain with the antigen binding fragment, as in pepsin digestion, then a divalent molecule is produced [$F(ab')_2$] which differs from the intact antibody by lack of an extended Fc portion. If the disulfide region is below the point of digestion, then the two heavy–light chain complexes that form the two antigen binding sites of an antibody are cleaved and released, forming individual dimeric fragments (Fab) containing one antigen binding site each (see Figure 20.4, discussed previously).

Methods for producing immobilized papain or pepsin for antibody fragmentation can be found in Hermanson *et al*. (1992). The following protocol describes the use of pepsin to cleave IgG molecules at the C-terminal side of the inter-heavy-chain disulfides in the hinge region, producing a bivalent antigen binding fragment, $F(ab')_2$, with a molecular weight of about 105,000 (Figure 20.10). Using this enzyme, most of the Fc fragments undergo extensive degradation and cannot be recovered intact.

Preparation of $F(ab')_2$ Fragments Using Pepsin

1. Equilibrate by washing 0.25 ml of immobilized pepsin (Thermo Fisher) with 4×1 ml of 20 mM sodium acetate, pH 4.5 (digestion buffer). Finally, suspend the gel in 1 ml of digestion buffer.
2. Dissolve 1–10 mg of IgG in 1 ml digestion buffer and add it to the gel suspension.
3. Mix the reaction slurry in a shaker at 37°C for 2–48 hours. The optimal time for complete digestion varies depending on the IgG subclass and species of origin. Mouse IgG1 antibodies are usually digested within 24 hours, human antibodies are fragmented in 12 hours, whereas some minor subclasses (e.g., mouse IgG2a) require a full 48-hour digestion period.
4. After the digestion is complete, add 3 ml of 10 mM Tris–HCl, pH 8.0, to the gel suspension. Separate the gel from the antibody solution using filtration or by centrifugation.
5. Apply the fragmented IgG solution to an immobilized protein A column containing 2 ml of gel (Thermo Fisher) that was previously equilibrated with 10 mM Tris–HCl, pH 8.0.

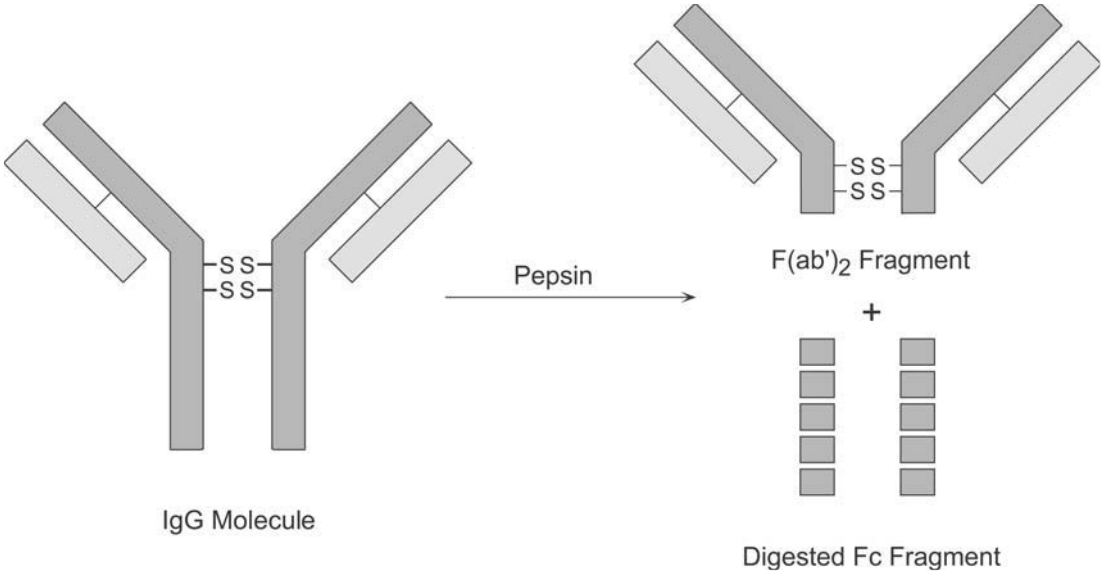

Figure 20.10 Digestion of IgG class antibodies with pepsin results in heavy chain cleavage below the disulfide groups in the hinge region. The bivalent fragments that are formed are called $F(ab')_2$. The remaining Fc region normally is severely degraded into smaller peptide fragments.

6. After the sample has entered the gel, wash the column with 10 mM Tris–HCl, pH 8.0, while collecting 2 ml fractions. The fractions may be monitored for protein by measuring absorbance at 280 nm. The protein peak eluting unretarded from the column is $F(ab')_2$.
7. Bound Fc or Fc fragments and any undigested IgG may be eluted from the column with 0.1 M glycine, pH 2.8.

Similarly, immobilized papain may be used to generate Fab fragments from immunoglobulin molecules. Papain is a sulfhydryl protease that is activated by the presence of a reducing agent. Cleavage of IgG occurs above the disulfides in the hinge region, creating two types of fragments, two identical Fab portions and one intact Fc fragment (Figure 20.11). For preparation of the immobilized papain gel used in the following protocol, see Hermanson *et al.* (1992). The gel also is commercially available from Thermo Fisher.

Preparation of Fab Fragments Using Papain

1. Wash 0.5 ml of immobilized papain (Thermo Fisher) with 4×2 ml of 20 mM sodium phosphate, 20 mM cysteine–HCl, 10 mM EDTA, pH 6.2 (digestion buffer), and finally suspend the gel in 1.0 ml of digestion buffer.
2. Dissolve 10 mg of human IgG solution in 1.0 ml of digestion buffer and add it to the immobilized papain gel suspension.
3. Mix the gel suspension in a shaker at 37°C for 4–48 hours. Maintain the gel in suspension during mixing. The optimal time for complete digestion varies depending on the IgG subclass

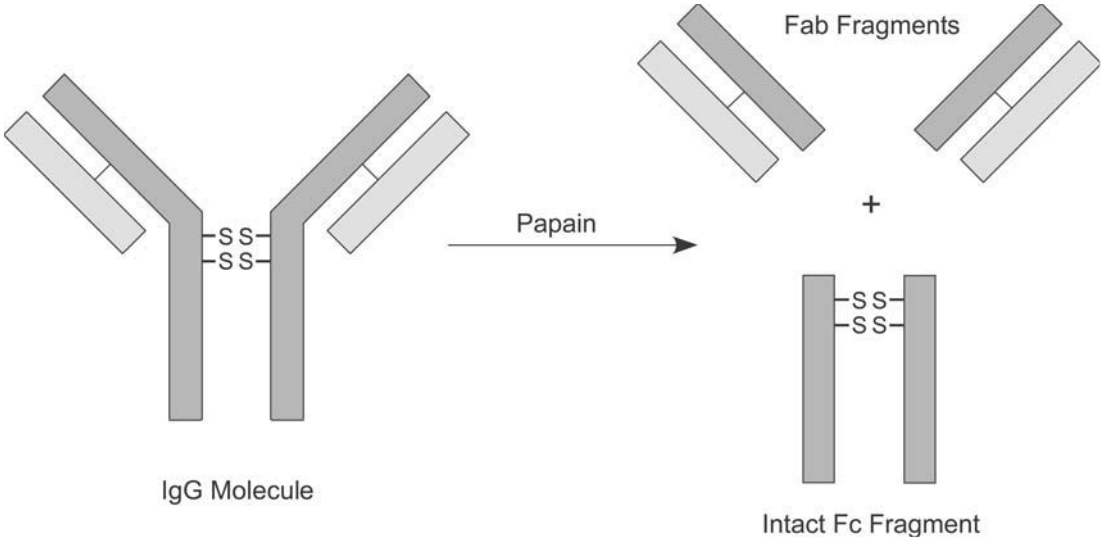

Figure 20.11 Papain digestion of IgG antibodies primarily results in cleavage in the hinge region above the interchain disulfides. This produces two heavy–light chain pairs, called Fab fragments, each containing one antigen binding site. The Fc region normally can be recovered intact.

and species of origin. Mouse IgG1 antibodies are usually digested within 27 hours, whereas other mouse subclasses require only 4 hours; human antibodies are fragmented in 4 hours (IgG1 and IgG3), 24 hours (IgG4), or 48 hours (IgG2); whereas bovine, sheep, and horse antibodies are somewhat resistant to digestion and require a full 48 hours.

4. After the required time of digestion, add 3.0 ml 10 mM Tris–HCl buffer, pH 8.0, to the gel suspension, mix, and then separate the digest solution from the gel by filtration or centrifugation at 2,000 g for 5 minutes.
5. Apply the supernatant liquid to an immobilized protein A column (2 ml gel; Thermo Fisher) which was previously equilibrated by washing with 20 ml of 10 mM Tris–HCl buffer, pH 8.0.
6. After the sample has entered the gel bed, wash the column with 15 ml of 10 mM Tris–HCl buffer, pH 8.0, while 2.0 ml fractions are collected. Monitor the fractions for protein by their absorbance at 280 nm. The protein eluted unretarded from the column is purified Fab.
7. Elute Fc and undigested IgG bound to the immobilized protein A column with 0.1 M glycine–HCl buffer, pH 2.8.

Conjugation of these fragments with enzymes is done using similar methods to those previously discussed for intact antibody molecules. $F(ab')_2$ fragments may be selectively reduced in the hinge region with DTT, TCEP, or MEA using the identical protocols outlined for whole antibody molecules (Chapter 1, Section 4.1, and Section 1.1, this chapter). Mild reduction results in cleaving the disulfides holding the heavy chain pairs together at the central portion of the fragment, thus creating two F(ab′) fragments each containing one antigen binding site (Figure 20.12).

The amine groups on these fragments also may be modified with thiolating agents, such as SATA or 2-iminothiolane, to create sulfhydryl residues suitable for coupling to maleimide-activated enzymes (Section 1.1, this chapter) (Figure 20.13). Amine groups further may be utilized

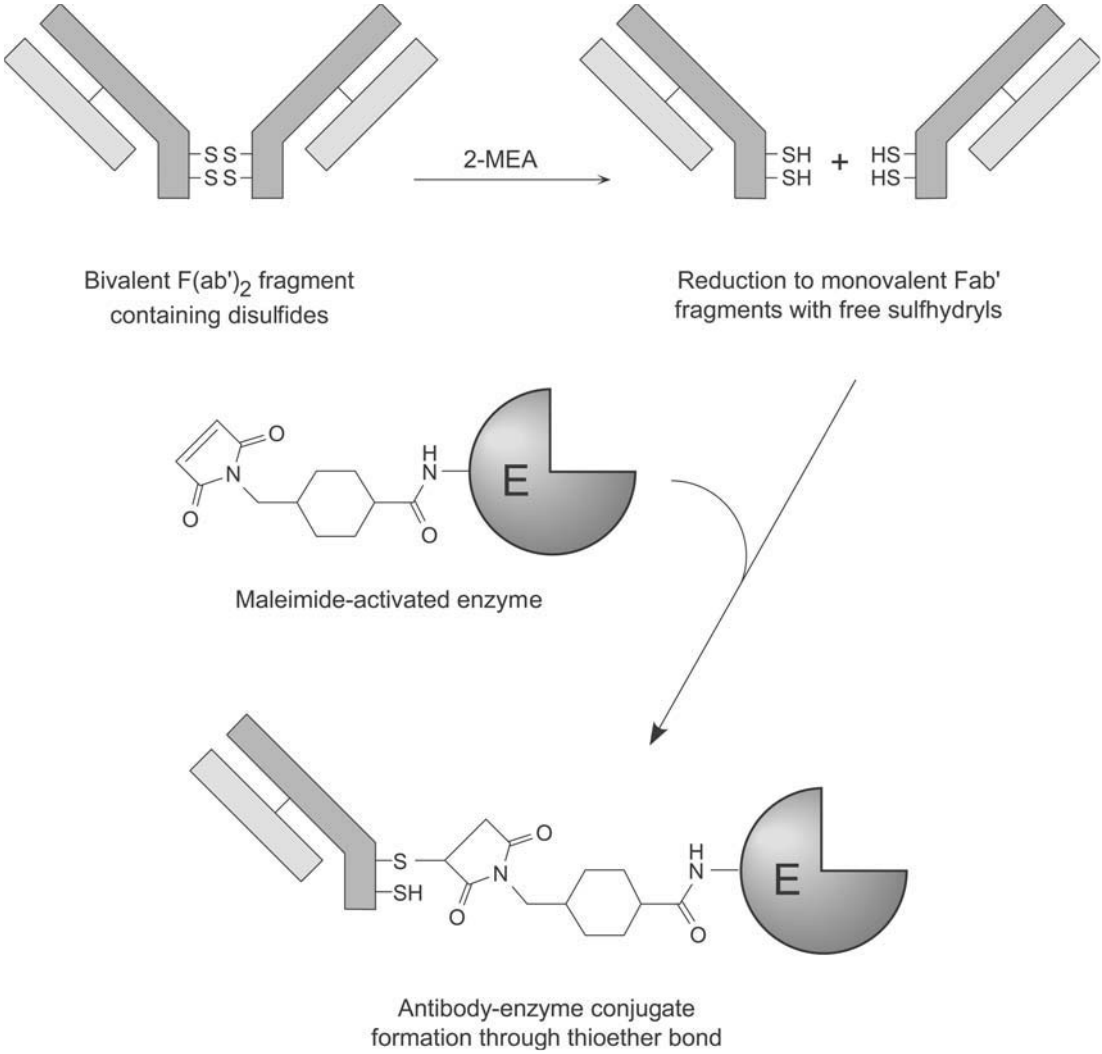

Figure 20.12 $F(ab')_2$ fragments produced by pepsin digestion of IgG can be reduced at their heavy chain disulfides using a reducing agent, such as MEA, DTT, or TCEP. Conjugation then can be done with a maleimide-activated enzyme to produce low-molecular-weight complexes linked by thioether bonds.

in reductive amination coupling to periodate-oxidized glycoproteins, such as in the protocol outlined for HRP conjugation, previously (Section 1.3, this chapter) (Figure 20.14). Successful periodate oxidation of the fragments themselves, however, may not be possible unless they contain carbohydrate in the antigen binding region, which is true for some polyclonal antibodies. Finally, glutaraldehyde-mediated conjugation techniques will work with antibody fragments, but are not recommended due to the reasons discussed in Section 1.2, this chapter.

The primary goal of any of these conjugation schemes using antibody fragments is to maintain the activity of the antigen binding site while limiting the size of the final complex with a

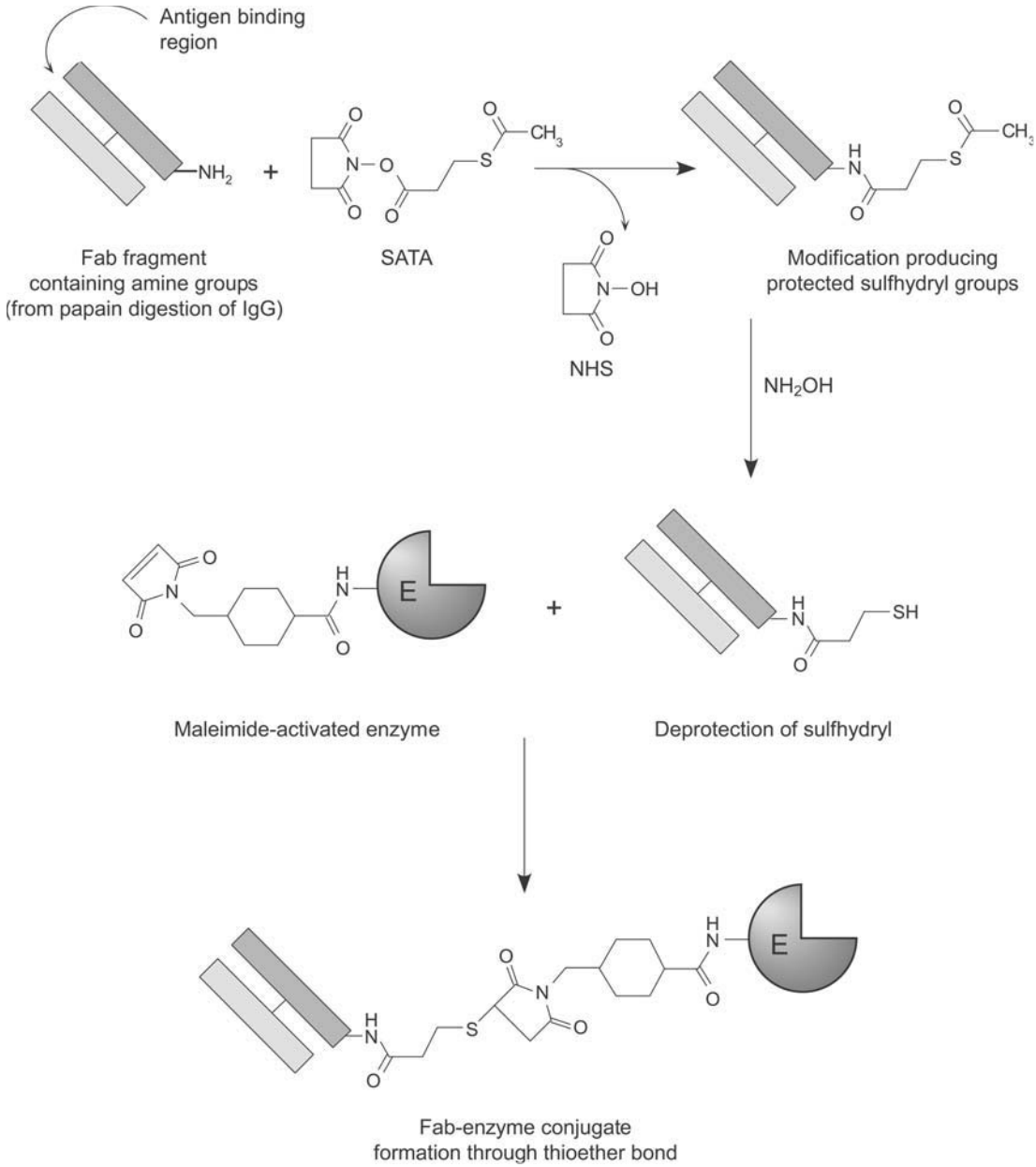

Figure 20.13 The thiolation reagent SATA can be used to create sulfhydryl groups on Fab fragments. After deprotection of the acetylated thiol of SATA with hydroxylamine, conjugation with a maleimide-activated enzyme can take place, producing thioether linkages.

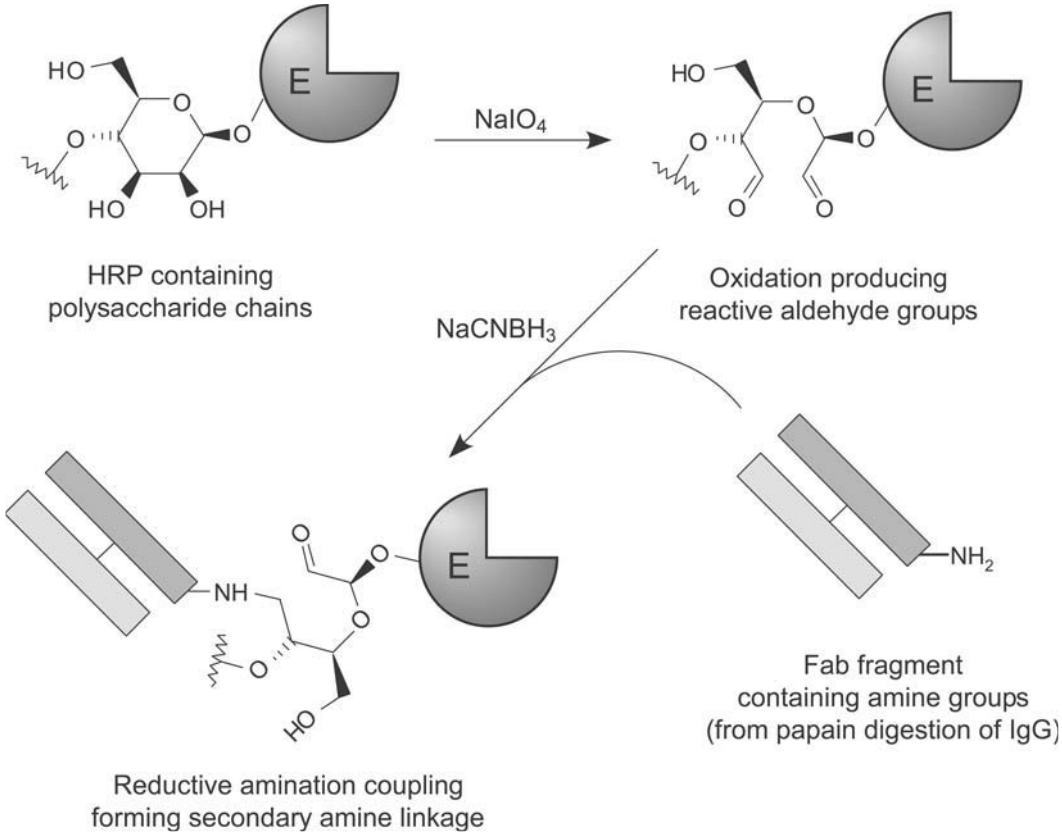

Figure 20.14 Periodate oxidation of HRP creates aldehyde groups on the carbohydrate chains of the enzyme. Reaction with a Fab fragment then may be done using reductive amination to produce a lower-molecular-weight complex than would be obtained using intact IgG antibodies.

second molecule. The use of heterobifunctional crosslinkers such as SMCC or reductive amination techniques allows sufficient control over the process to realize these goals.

1.5. Removal of Unconjugated Enzyme from Antibody–Enzyme Conjugates

Conjugates of antibodies and enzymes are essential components in immunoassay and detection systems. In the preparation of such conjugates, a molar excess of enzyme typically is crosslinked to a specific antibody to obtain a complex of high activity. The result of this ratio is excess enzyme left unconjugated after completion of the reaction. The unconjugated enzyme confers nothing to the utility of the final product and can be detrimental by contributing to increased backgrounds in assay procedures. The removal of this free enzyme component may be advantageous to improving the resultant signal-to-noise ratio in some immunoassays. Commercial preparations of antibody–enzyme conjugates usually are not purified to remove unconjugated enzyme.

Frequently, the major proteinaceous part of these products is not active conjugate, but leftover enzyme that contributes nothing to the immunochemical activity of what was purchased.

Boorsma and Kalsbeek (1976) state that unconjugated HRP must be removed from antibody–enzyme conjugates to obtain optimal staining in immunoassay procedures. This is especially true in blotting techniques and cytochemical staining where free enzyme may become entrapped nonspecifically within the membrane or cellular structures. The presence of this unconjugated enzyme leads to diffuse substrate noise that can obscure the immunospecific signal.

Several methods may be used to purify an antibody–enzyme conjugate and remove unconjugated enzyme. For instances where the enzyme molecular weight is significantly different than the conjugate molecular weight, separation may be achieved by gel filtration chromatography. Using the proper support with an exclusion limit and separation range able to accommodate all the proteins in the sample, the conjugate peak will elute before the enzyme peak, thus providing an efficient way of removing free enzyme. However, gel filtration procedures can be time consuming and of relatively low capacity for the amount of gel required. In addition, separation of higher-molecular-weight enzymes from antibody conjugates, such as in the case of AP (MW 140,000), is considerably less efficient or impossible. Gel filtration separation also becomes a problem if the conjugate itself consists of a broad range of molecular weights, as is often true when glutaraldehyde is used as the crosslinking agent.

The most effective methods of removing unconjugated enzyme all make use of affinity chromatography systems using specific ligands that can interact with the antibody portion of the conjugate. Thus, the supports retain any unconjugated antibody (usually in very low percentage when the enzyme is reacted in excess) as well as the antibody–enzyme conjugate produced from the crosslinking reaction. The unconjugated enzyme, however, passes through such affinity columns unretarded. Two main methods are discussed below: (1) affinity chromatography which makes use of immobilized immunoglobulin binding proteins or immobilized antigen molecules having specificity for the antibody used in the conjugate and (2) nickel-chelate affinity chromatography which binds the Fc region of antibody molecules.

The use of immunoaffinity techniques (whether antigen specific or immunoglobulin binding proteins such as protein A) allows strong binding of the antibody conjugate, but have the significant disadvantage of requiring elution conditions that are often too severe for maintaining activity of the antibody or enzyme components. By contrast, nickel-chelate affinity techniques give excellent binding of the conjugate while allowing free enzyme to pass through the gel unretarded. It also has the significant advantage of having mild elution conditions which preserve the activity of the conjugate.

Immunoaffinity Chromatography

Immunoaffinity chromatography makes use of immobilized antigen molecules to bind and separate specific antibody from a complex mixture. After the preparation of an antibody–enzyme conjugate, the antibody binding capability of the crosslinked complex toward its complementary antigen ideally remains intact. This highly specific interaction can be used to purify the conjugate from excess enzyme if the antibody and enzyme can survive the conditions necessary for binding and elution from such a column. Binding conditions typically are mild physiological pH conditions which cause no difficulty. However, many elution conditions require acidic or basic conditions or the presence of a chaotropic agent to deform the antigen binding site. Sometimes these conditions can irreversibly damage the antigen binding recognition capability

of the antibody or denature the active site of the enzyme, thus diminishing enzymatic activity. Activity losses for both the antibody and enzyme can be severe under such circumstances.

Another potential disadvantage of an immunoaffinity separation is the assumed abundance of the purified antigen in sufficient quantities to immobilize on a chromatography support. Protein antigens should be immobilized at densities of at least 2–3 mg/ml of affinity gel to produce supports of acceptable capacity for binding antibody. Often, the antigen is too expensive or scarce to obtain in the amounts needed.

However, if the antigen is abundant and inexpensive and the antibody–enzyme complex will survive the associated elution conditions, then immunoaffinity chromatography can provide a very efficient method of purifying a conjugate from excess enzyme. This method also assures that the recovered antibody still retains its ability to bind specific target molecules (i.e., the antigen binding site was not blocked during conjugation). The preparation of immunoaffinity supports can be found in Hermanson *et al.* (1992). A suggested method for performing immunoaffinity chromatography follows.

Protocol

1. Equilibrate the immunoaffinity column with 50 mM Tris, 0.15 M NaCl, pH 8.0 (binding buffer). Wash with at least 5 column volumes of buffer. The amount of gel used should be based on the total binding capacity of the support. A determination of binding capacity can be done by overloading a small-scale column, eluting, and measuring the amount of conjugate that bound. Such an experiment may be coupled with a determination of conjugate viability for using immunoaffinity as the purification method. The final column size should represent an amount of gel capable of binding at least 1.5 times more than the amount of conjugate that will be applied.
2. Apply the conjugate to the column in the binding buffer while taking 2 ml fractions.
3. Wash with binding buffer until the absorbance at 280 nm decreases back to baseline. The unbound protein flowing through the column will consist of mainly unconjugated enzyme. Some conjugate may flow through also if some of the conjugate is inactive or the column is overloaded.
4. Elute the bound conjugate with 0.1 M glycine, 0.15 M NaCl, pH 2.8, or another suitable elution buffer. A neutral pH alternative to this buffer is the Gentle Elution Buffer from Thermo Fisher. If acid pH conditions are used, immediately neutralize the fractions eluting from the column by the addition of 0.5 ml of 1 M Tris, pH 8.0, per fraction.

Nickel-Chelate Affinity Chromatography

Metal-chelate affinity chromatography is a powerful purification technique whereby proteins or other molecules can be separated based upon their ability to form coordination complexes with immobilized metal ions (Porath *et al.*, 1975; Lonnerdal and Keen, 1982; Porath and Belew, 1983; Porath and Olin, 1983; Sulkowski, 1985; Kagedal, 1989). The metal ions are stabilized on a matrix through the use of chelating compounds which usually have multivalent points of interaction with the metal atoms. To form useful affinity supports, these metal ion complexes must have some free or weakly associated and exchangeable coordination sites. These exchangeable sites then can form complexes with coordination sites on proteins or other molecules. Substances that are able to interact with the immobilized metals will bind and be retained on

the column. Elution is typically accomplished by one or a combination of the following options: (1) lowering of pH, (2) raising the salt strength, and/or (3) the inclusion of competing chelating agents such as EDTA or imidazole in the buffer.

Sorensen (1993) reported that a nickel-chelate affinity column will specifically bind IgG class immunoglobulins while allowing certain enzymes to pass through the gel unretarded (Thermo Fisher). This phenomenon allows the separation of antibody–enzyme complexes containing, in particular, HRP or AP conjugated to common polyclonal or monoclonal antibodies. The nickel-chelate column binds the conjugate through the Fc region of the associated antibody, even if enzyme molecules are covalently attached. Any unconjugated enzyme will pass through the affinity column unretarded (Figure 20.15).

Elution of the bound antibody–enzyme conjugate occurs by only a slight shift in pH to acidic conditions or through the inclusion of a metal-chelating agent like EDTA or imidazole in the binding buffer. Either method of elution is mild compared to most immunoaffinity separation techniques (discussed in the previous section). Thus, purification of the antibody–enzyme complex can be done without damage to the activity of either component.

One limitation to this method should be noted. If the antibody–enzyme conjugate is prepared using antibody fragments such as Fab or $F(ab')_2$, then nickel-chelate affinity chromatography will not work, since the requisite Fc portion of the antibody necessary for complexing with the metal is not present.

The preparation of a metal-chelate affinity support containing iminodiacetic acid functionalities may be found in Hermanson *et al.* (1992), or purchased from a commercial source. Any metal-chelate resin designed to bind His-tagged fusion proteins also will work well in this procedure. The following protocol is adapted from the instructions accompanying the nickel-chelate support. Thermo Fisher offers a kit based on this technology for the purpose of removing unconjugated enzyme from antibody–enzyme conjugates (called the FreeZymep Conjugate Purification Kit).

Protocol

1. Pack a column containing an immobilized iminodiacetic acid support (or another chelating agent designed to bind His-tagged proteins) (Thermo Fisher). The column size should be no less than 1.5 times that required to bind the anticipated amount of conjugate to be applied. The maximal capacity of such a column for binding antibody can be up to 50 mg/ml gel, however best results are obtained if no more than 10–20 mg/ml of conjugate is applied.
2. Dissolve 50 mg of nickel ammonium sulfate per ml of deionized water. Apply 1 ml of nickel solution per ml of gel to the column. *Note*: The metal salt and all solutions containing it should be considered hazardous waste and disposed of according to relevant environmental regulations.
3. Wash the column with 10 volumes of water, then equilibrate the support with 2 volumes of 10 mM sodium phosphate, 0.15 M NaCl, pH 7.0 (binding buffer).
4. Dissolve or dialyze the conjugate into binding buffer. Apply the conjugate solution to the column while collecting 2 ml fractions.
5. Continue to wash the gel with 0.15 M NaCl (saline solution) until the absorbance at 280 nm is down to baseline. The protein eluting from the column at this point is unconjugated enzyme.

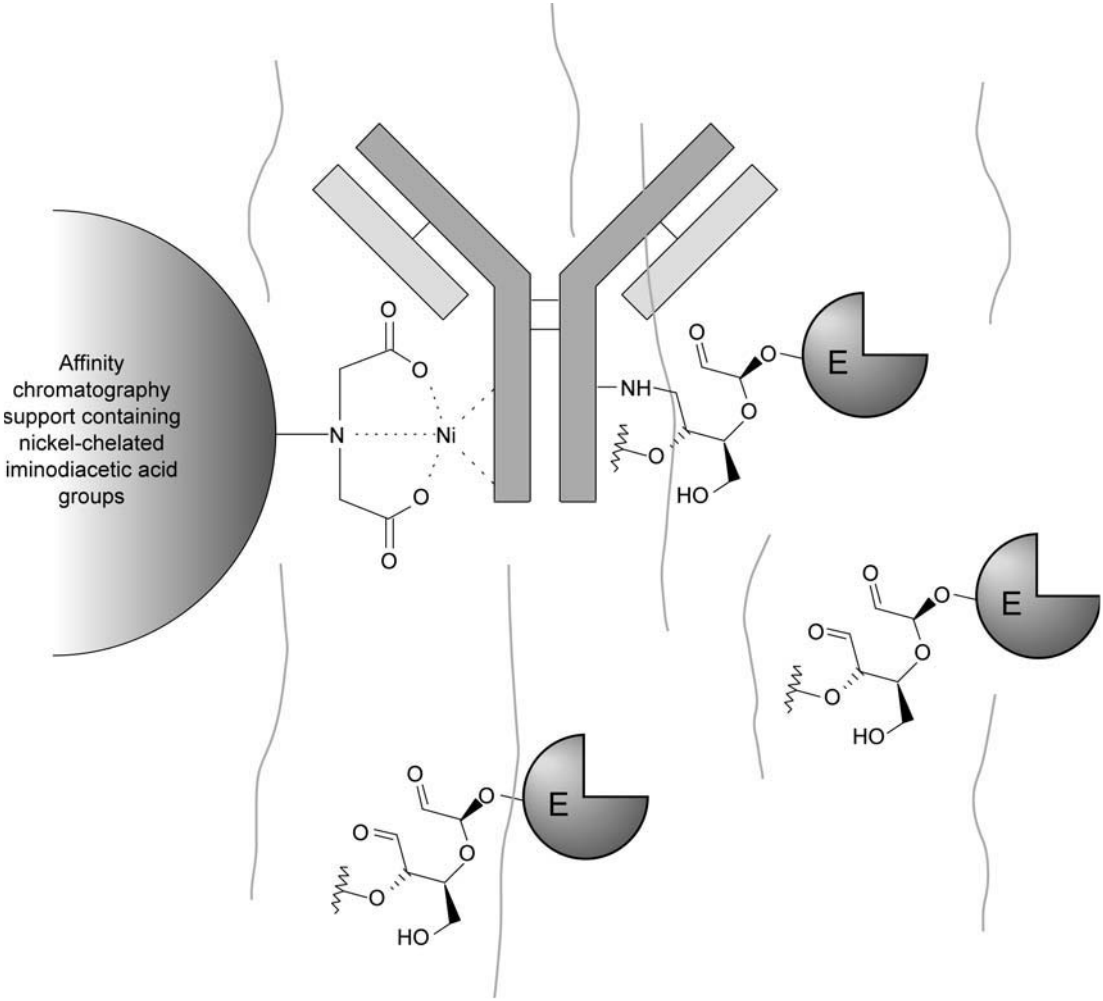

Figure 20.15 An affinity chromatography support containing iminodiacetic acid groups chelated with nickel may be used to remove excess enzyme after reactions to produce antibody–enzyme conjugates. The nickel chelate binds to the antibody in the Fc region, retaining the conjugate while allowing free enzyme to pass through the gel unretarded.

6. Elute the bound conjugate with 0.1 M sodium acetate, 0.5 M NaCl, pH 5.0. Pool the fractions containing protein, and dialyze the conjugate into 10 mM sodium phosphate, 0.15 M NaCl, pH 7.0, or other suitable storage buffers.

2. Preparation of Labeled Antibodies

In addition to labeling immunoglobulins with enzymes to provide detectability through their catalytic action on a substrate, antibody molecules also can be labeled or tagged with small

compounds that can provide detectable properties. The specificity of the antibody then can be used to bind unique antigenic determinants, while the attached tag supplies the properties necessary for detection. Such small chemical labels typically are one of two types: intense fluorophores or unstable, radioactive isotopes.

Radiolabeling antibodies with ^{125}I form the basis for highly sensitive radioimmunoassays (RIA) that were first developed in the early days of immunoglobulin-mediated testing. The use of radioisotopes in tagging antibodies is used less often today for *in vitro* immunoassays due to the hazards associated with handling and disposal of radioactive compounds. However, isotopes other than ^{125}I are becoming very important as monoclonal labels for use in *in vivo* diagnostic or therapeutic procedures for cancer therapy or detection. In addition, a radiolabel has distinct advantages over other chemical tags. It is not influenced by conformational changes within the antibody molecule or by changes in its chemical environment as enzymes or labels with unique spectral characteristics can be. Thus, radiolabels still can provide a means of detection equal to or exceeding the most sensitive and reliable tags now available.

Another form of label often used to tag antibody molecules is chemical modification with a reagent terminating in a biotin group. Biotinylation (Chapter 11) creates an affinity handle on the immunoglobulin with the ability to bind strongly avidin or streptavidin in one of the most tightly held noncovalent interactions known. With a dissociation constant (K_d) on the order of 1.3×10^{-15}, the avidin–biotin interaction can be used to detect biotinylated molecules with extreme sensitivity. In this type of system, instead of the antibody being labeled, the avidin (or streptavidin) molecules are modified to contain the detection complex—consisting of enzyme, fluorophore, or radiolabel. Interaction of the biotinylated antibody with its targeted antigen is amplified and detected by addition of such labeled avidin or streptavidin reagents.

The following three sections describe the preparation and properties of fluorescent, radiolabeled, and biotinylated antibodies.

2.1. Fluorescently Labeled Antibodies

Antibody molecules can be labeled with any one of more than a dozen different fluorescent probes currently available from commercial sources. Each probe option has its own characteristic spectral signals of excitation (or absorption) and emission (or fluorescence). Many derivatives of these fluorescent probes possess reactive functionalities convenient for covalently linking to antibodies and other molecules. Each of the main fluorophore families contains at least a few different choices in coupling chemistry to direct the modification reaction to selected functional groups on the molecule to be labeled. These choices include amine-reactive, sulfhydryl-reactive, and carbonyl-reactive. Examples of some of the more popular varieties of fluorescent probes can be found in Chapter 9.

In addition to the wide range of commercial probes, many other fluorescent molecules have been synthesized and described in the literature. Only a handful, however, are generally used to label antibody molecules. Perhaps the most common fluorescent tags with application to immunoglobulin assays are reflected in the main derivatives produced by the prominent antibody manufacturing companies. These include derivatives of cyanine dyes, fluorescein, rhodamine, Texas red, aminomethylcoumarin (AMCA), and phycoerythrin. Figure 20.16 shows the reaction of fluorescein isothiocyanate (FITC), one of the most common fluorescent probes, with an antibody molecule.

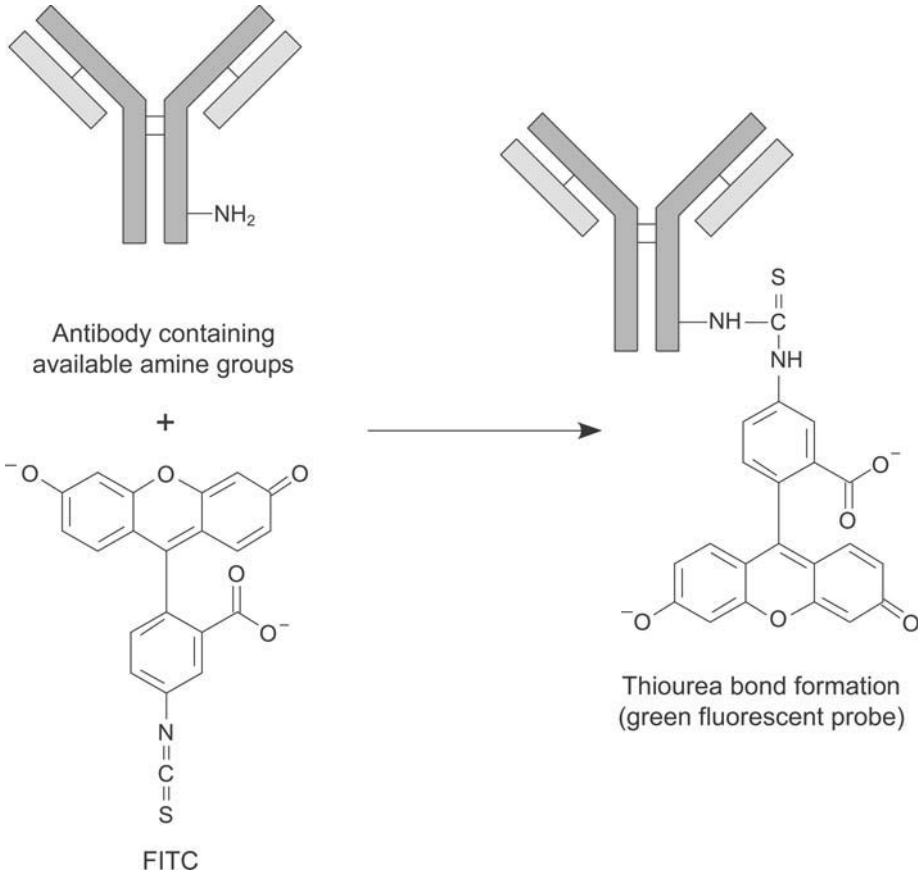

Figure 20.16 FITC may be used to label amine groups on antibody molecules, forming isothiourea bonds.

To a large degree, standardization has occurred in the use of these fluorescent probes due to the large literature documentation available on their successful application to antibody-based assays. As a result of this, instrumentation has become widely available for measuring their fluorescence signals, including standard filter selections which match common excitation and emission wavelengths. Such fluorescently labeled antibodies can be used in immunohistochemical staining (Osborn and Weber, 1982), in flow cytometry or cell sorting techniques (Ormerod, 1990; Watson, 2004), for tracking and localization of antigens, and in various double-staining methods (Kawamura, 1977). Extensive use of fluorescent antibodies in microscopy, pathology, and high content screening for drug discovery has made fluorescently labeled antibodies the most common antibody derivatives used in immunochemical detection techniques (Lichtman and Conchello, 2005).

In choosing a fluorescent tag, the most important factors to consider are good adsorption (high extinction coefficient), stable excitation without photobleaching, and efficient, high quantum yield of fluorescence. Some fluorophores, such as fluorescein, exhibit rapid fluorescent quenching which lowers the quantum yield over time. Up to 50 percent of the fluorescent intensity observed on a fluorescein-stained slide can be lost within 1 month in storage. AMCA and

some cyanine dyes have much better stability, but all fluorophores lose some intensity upon exposure to light or upon storage. Exceptions to this rule are the use of fluorescent nanoparticles, such as silica (Chapter 14, Section 5) and quantum dots (Chapter 9, Section 10).

In some cases, the preparation of a fluorescently labeled antibody is not even necessary. Particularly, if indirect methods are used to detect antibody binding to antigen, then preparing a fluorescently labeled primary antibody is not needed. Instead, the selection from a commercial source of a labeled secondary antibody having specificity for the species and class of primary antibody to be used is all that is required. However, if the primary antibody needs to be labeled and it is not manufactured commercially, then a custom labeling procedure will have to be done.

Generalized protocols for the attachment of these fluorophores to protein molecules, including antibodies, can be found in Chapter 9 and Chapter 14, Section 5. The main consideration for the modification of immunoglobulins is to couple these probes at an optimal level to allow good detectability without high backgrounds. Too low a substitution level and the response of the fluorophore will yield low signal strength and poor sensitivity. Too high a substitution level and the fluorophore may self-quench through energy transfer, decrease the antibody's ability to bind target molecules by blocking the antigen binding sites, or cause nonspecific interactions resulting in high background or noise levels. In some cases, trial and error will be required to optimize this process.

For other examples of antibody labeling protocols see Goding (1976) and Harlow and Lane (1988).

2.2. Radiolabeled Antibodies

The attachment of a radioactive label onto an antibody molecule provides a powerful means of detection in immunoassay procedures, tracking of analytes, for *in vivo* diagnostic procedures, and, more recently, for the detection or therapy of numerous types of cancers. Originally, radiolabeling of antibodies merely meant modifying tyrosine residues with ^{125}I. Now, a number of different radioactive elements are being attached, both covalently and through specialized chelating compounds to provide imaging capabilities for the detection of primary tumors and metastases (Order, 1989).

Radioiodination can be done using any one of a number of techniques. Most of the procedures utilize ^{125}I as the unstable isotope of choice for *in vitro* use due to its easy availability, comparably long 60-day half-life, and relatively low-energy photon emissions. Radioactive ^{125}I usually is supplied as its sodium salt and must be oxidized to create an electrophilic species capable of modifying molecules. Commonly used oxidizing agents include chloramine-T, Iodogen, and Iodo-beads (Chapter 12). When used in direct labeling techniques with antibodies and other proteins, these oxidants cause an iodination reaction to occur at available tyrosine residues within the polypeptide chain. If tyrosine is important to antibody activity and cannot be labeled, then certain crosslinking or modification reagents containing an activated aromatic ring also may be iodinated to label at other functional sites within the protein molecule. An example of this technique is to use the Bolton–Hunter reagent (Chapter 12, Section 5) labeled with radioactive iodine to modify the primary amines within the antibody. This reagent also can be used to add an iodinatable site to molecules containing no tyrosine residues (Figure 20.17).

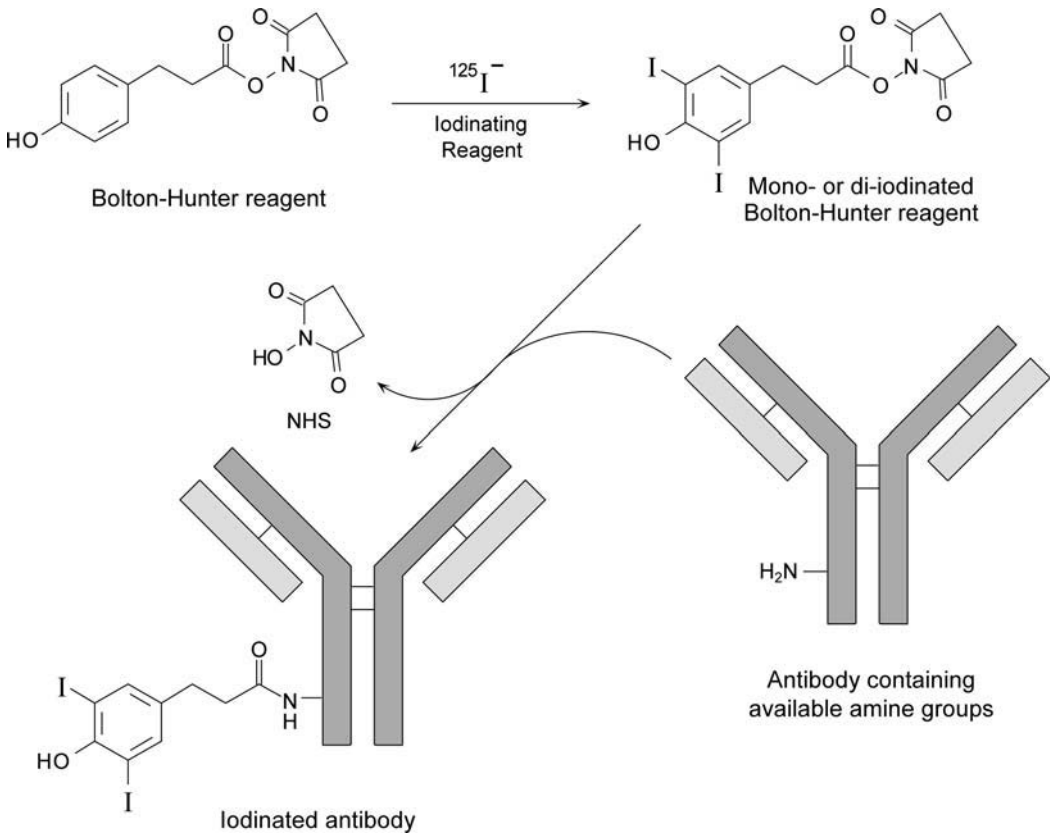

Figure 20.17 Bolton–Hunter reagent may be used to add radioactive iodine labels to antibody molecules by modification of amines.

Reagent options and protocols for the radioiodination of antibodies and other molecules may be found in Chapter 12.

Another method of adding a radioactive tag to antibodies is to use a chelating compound capable of complexing metal isotopes. One of the most frequently used chelating reagents is diethylenetriamine pentaacetic acid (DTPA) (Chapter 10, Section 1). The reagent contains two anhydride groups that can be used to modify primary amines in proteins and other molecules. The reaction process involves ring opening and the formation of an amide bond. Ring opening also creates up to four free carboxylate groups which, combined with the three nitrogen atoms in the chelator, are able to form str820ong coordination complexes with metals such as indium-111 (Figure 20.18). Monoclonals labeled with chelated isotopes can be used in targeting tumor cells *in vivo*. The detection sensitivity of radiolabeled antibodies has led to effective diagnostic procedures to monitor primary and secondary cancer growths. In addition, the intensity of radioactivity at the tumor site when labeled monoclonals are targeted therapeutically can be great enough to cause tumor cell death and remission.

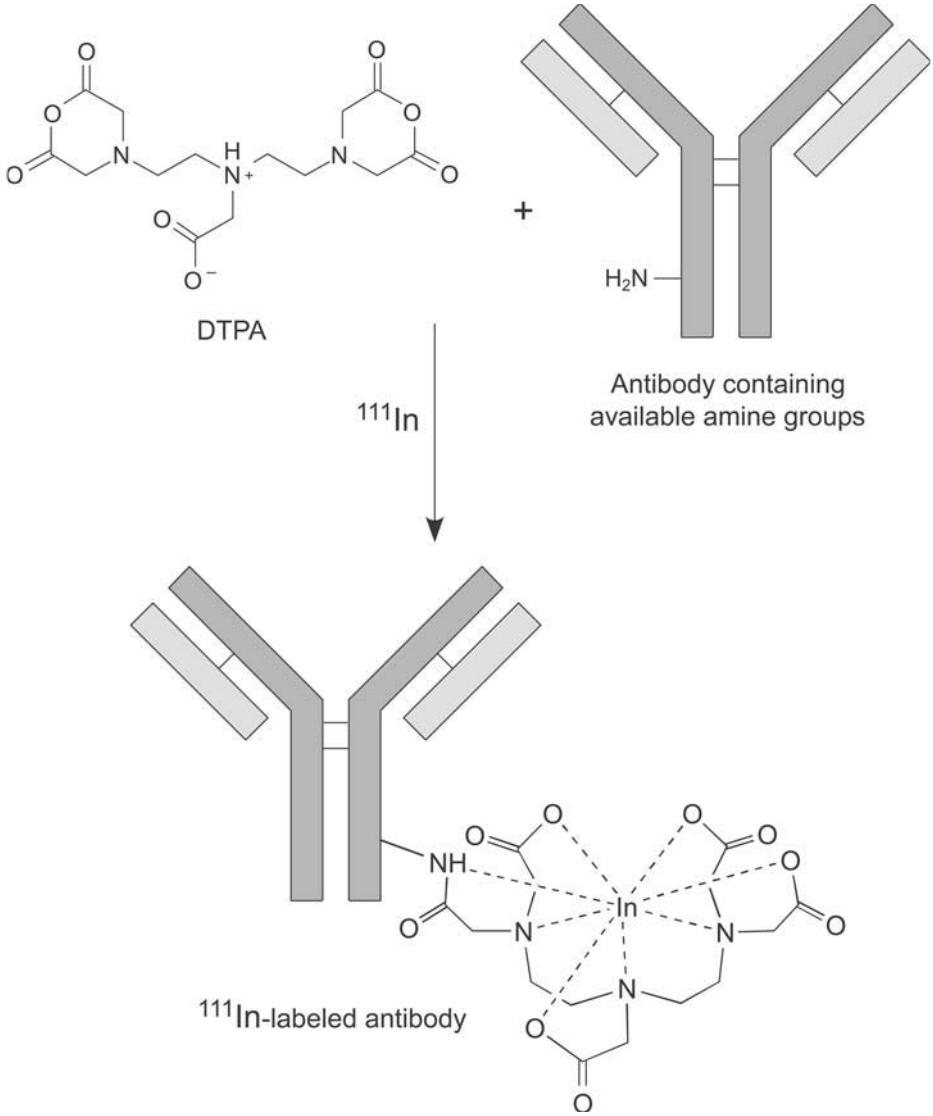

Figure 20.18 The bifunctional chelating reagent DTPA may be used to modify amine groups on antibody molecules, forming amide bond linkages. Indium-111 then may be complexed to the chelator group to create a radiolabeled-targeting reagent.

2.3. Biotinylated Antibodies

Another popular tag for use with immunoglobulins is biotin. Modification reagents that can add a functional biotin group to proteins, nucleic acids, and other molecules now come in many shapes and reactivities (Chapter 11). Depending on the reactive group present on the biotinylation

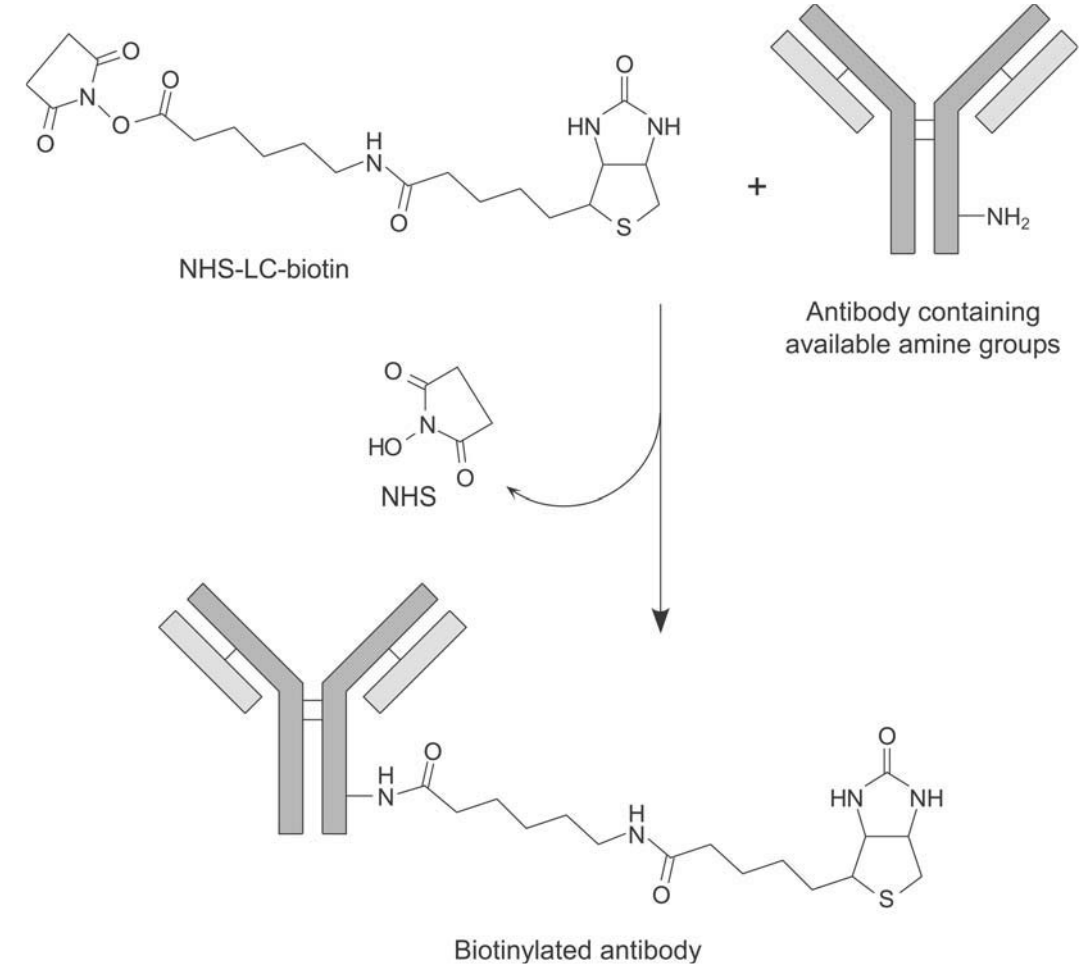

Figure 20.19 Biotinylated antibodies can be formed by reacting NHS-LC-biotin with available amine groups to create amide bonds.

reagent, specific functional groups on antibodies may be modified to create an affinity tag capable of binding avidin (or streptavidin) derivatives. Amines, carboxylates, sulfhydryls, and carbohydrate groups can be specifically targeted for biotinylation through the appropriate choice of reactive biotin compound. Figure 20.19 shows the biotinylation of an antibody with NHS-LC-biotin, one of the most common biotinylation reagents.

The presence of biotin labels on an antibody molecule provides multiple sites for the binding of avidin or streptavidin. If the biotin binding protein is in turn labeled with an enzyme, fluorophore, etc., then a very sensitive detection system is created. The potential for more than one labeled (strept)avidin to become attached to each antibody through multiple biotinylation sites provides an increase in detectability over antibodies directly labeled with a detectable tag.

Several assay designs that use the enhanced sensitivity afforded through biotinylated antibodies have been developed. Most of these systems use conjugates of avidin or streptavidin

with enzymes (such as HRP or AP), although other labels (such as fluorophores) can be used as well. In the simplest assay design, called the labeled avidin–biotin (LAB) system, a biotinylated antibody is allowed to incubate and bind with its target antigen. Next, an avidin–enzyme conjugate is introduced and allowed to interact with the available biotinylation sites on the bound antibody. Substrate development then provides the detectability necessary to quantify the antigen.

In a slightly more complex design, the bridged avidin–biotin system (BRAB) uses (strept) avidin's multiple biotin binding sites to create an assay potentially of higher sensitivity than that of the LAB assay. Again, the biotinylated antibody is allowed to bind its target, but next unmodified (strept)avidin is introduced to bind with the biotin binding sites on the antibody. Finally, a biotinylated enzyme is added to provide a detection vehicle. Since the bound (strept)avidin still has additional biotin binding sites available, the potential exists for more than one biotinylated enzyme to interact with each bound (strept)avidin molecule. In some cases, sensitivity can be increased over that of the LAB technique by using the bridging ability of avidin or streptavidin (Chapter 23, Section 2).

A modification on this theme can be used to produce one of the most sensitive enzyme-linked assay systems known. The ABC system (for avidin–biotin complex) increases antigen detectability beyond that possible with either the LAB or BRAB designs by forming a polymer of biotinylated enzyme and (strept)avidin before addition to an antigen-bound, biotinylated antibody. When avidin and a biotinylated enzyme are mixed together in solution in the proper proportion, the multiple binding sites on (strept)avidin create a high molecular weight, multimeric complex. If the biotinylated enzyme is not in large enough excess to block the binding sites on all the (strept)avidin molecules, then additional sites will still be available on this complex to bind a biotinylated antibody bound to its complementary antigen. The large complex provides multiple enzyme molecules to enhance dramatically the sensitivity of detecting antigen. Thus, the ABC procedure is currently among the highest-sensitivity methods available for immunoassay work.

More detailed discussions of avidin–biotin systems as well as the process of adding a biotin affinity group to proteins, nucleic acids, and other biomolecules can be found in Chapters 11 and 23.

21

Immunotoxin Conjugation Techniques

Monoclonal antibodies directed against tumor antigens may be used as targeting agents for conducting certain cytotoxic substances to malignant cells for selective killing. Numerous cell-surface markers are known to proliferate in human solid tumors (Boyer *et al.*, 1988; Carter *et al.*, 2004). The ability to raise mono-specific antibodies to these markers creates the capacity to target discretely the tumor, causing cell death while leaving healthy cells alone (Salmon, 1989; McCarron *et al.*, 2005).

The design of such cytotoxic antibodies is conceptually simple: attach a toxic substance or a mediator of toxicity to the appropriate monoclonal and you have a "magic bullet" that can find and eliminate the one-in-a-billion cells that have the requisite marker (Figure 21.1). The antibody provides the recognition and binding capacity, while the associated toxic component effects cellular alterations leading to cell death (Pastan *et al.*, 2006).

The approach to constructing antibody immunoconjugates for cancer has taken a number of forms (Vogel, 1987). One of the first designs used conjugates of monoclonal antibodies with toxins that were able to block protein synthesis at the ribosome level inside the cell (Lord *et al.*, 1988). Other conjugate forms used radioactive labels that killed cells by over-exposure to radiation in proximity to where antibody docking occurred (Order, 1989). Drug conjugates also were constructed that combined the known benefits of chemotherapy with the targeting capability of monoclonals (Reisfeld *et al.*, 1989; Willner *et al.*, 1993). The expected result was the effective concentration of the chemotherapeutic agent at the location of the tumor—hopefully eliminating or minimizing the side-effects of traditional chemotherapy. In addition, conjugates of monoclonals with certain biological modulators such as lymphokines or growth factors were tried to affect malignant cell viability (Obrist *et al.*, 1988).

Some immunoconjugates utilize intermediate carrier systems consisting of polymeric molecules such as polysaccharides, particularly dextran (Hurwitz *et al.*, 1978, 1980, 1983a, b, 1985; Manabe *et al.*, 1983; Sela and Hurwitz, 1987). The activated dextran is crosslinked to both the monoclonal and the cytotoxic agent, providing multivalent conjugation sites to create larger complexes (Section 2.3, this chapter and Chapter 25, Section 2). Liposomes can be used in similar fashion by anchoring the antibody to its outer surface and charging the vesicle with cytotoxic compounds (Gregoriadis, 1984; Matthay *et al.*, 1986; Singhal and Gupta, 1986; Ho *et al.*, 1986). See Chapter 22 for a survey of liposome conjugation techniques. Also, dendrimer

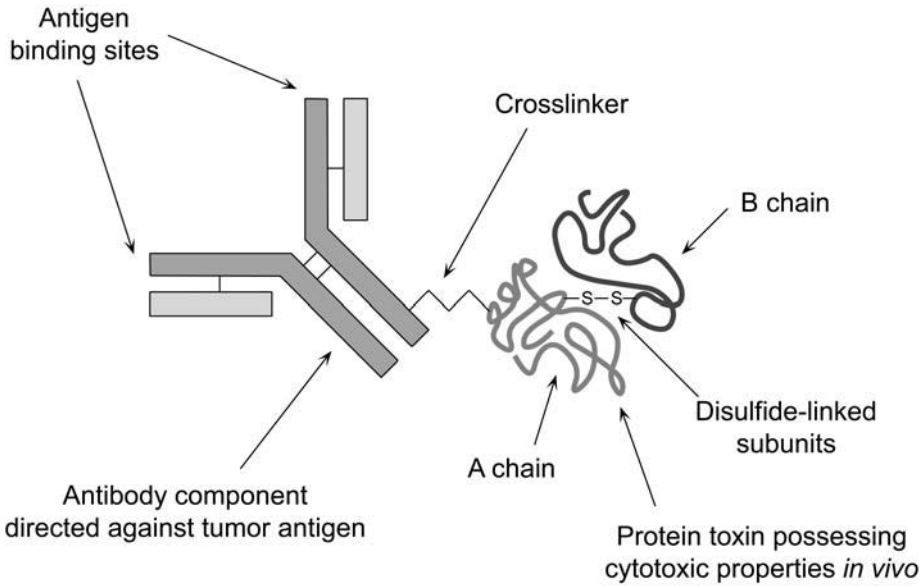

Figure 21.1 The basic design of an immunotoxin conjugate consists of an antibody-targeting component crosslinked to a toxin molecule. The complexation typically includes a disulfide bond between the antibody portion and the cytotoxic component of the conjugate to allow release of the toxin intracellularly. In this illustration, an intact A–B toxin protein provides the requisite disulfide, but the linkage also may be designed into the crosslinker itself.

conjugates with antibody-targeting molecules and cytotoxic components have been used to create multivalent immunotoxin conjugates (Chapter 7).

Other systems were designed to use two-stage approaches where an antibody was conjugated with an intermediate agent, which, when combined with another factor, could elicit cytotoxicity. For instance, enzyme conjugates with monoclonals have been used that could transform an inactive pro-drug into a chemotactic agent *in vivo* (Senter *et al*., 1988). Monoclonals also could be tagged with a biotin label and a secondary, avidin–toxin conjugate used to target the antibody once it bound the tumor cell. Two-stage radiative treatment also has been tried through the use of boron neutron capture therapy (Holmberg and Meurling, 1993). In this case, a carrier molecule is modified to contain ^{10}B. After administration *in vivo* to target tumor cells, neutron bombardment yields an unstable intermediate ^{11}B which immediately undergoes a fission reaction to yield ^{7}Li and ^{4}He. The induced radiation then kills the cells.

Tumor-targeting conjugates which use biospecific agents other than monoclonal antibodies have been developed as well. The targeting component in these systems consists of any molecule that can function as a ligand having specific affinity for some receptor molecule on the surface of the tumor cells. Such an affinity molecule might be a hormone (typically called hormonotoxins; Singh *et al*., 1989, 1993), a growth factor, an antigen specific for binding particular antibodies projecting from B cell surfaces, transferrin, α_2-macroglobulin, or anything else able to specifically interact with the targeted tumor cells.

It became apparent very early in the development of such agents that their conception and design was much easier to imagine than to successfully implement. Monoclonal antibodies used

in vitro could easily detect antigen molecules in complex mixtures with very little nonspecificity or cross-reactivity. Very quickly following the invention of hybridoma technology, monoclonals were employed in numerous diagnostic assays. Their use as therapeutic agents *in vivo*, however, was complicated by the body's natural immune response designed to prevent the invasion of foreign substances. At the time of this writing, there only are six unconjugated antibody therapeutics approved for use in the U.S.A along with two radioimmunoconjugates, which use a monoclonal antibody labeled with a radioactive metal ion. The only immunotoxin conjugate employing an antibody–phytotoxin conjugate is Mylotarg, a conjugate of an anti-CD33 monoclonal with a calicheamicin derivative (an anti-tumor antibiotic) (Bross *et al*., 2001).

A major problem in the use of immunotoxins is that injection of conjugates prepared from mouse monoclonals usually results in antibody production against the foreign protein. Sometimes allergic reactions can further complicate the side-effects, making continued therapy infeasible. Most often, however, induced host immunoglobulins will quickly bind the immunoconjugate and remove it from the circulatory system. Instead of finding the targeted tumor cells, the immunotoxin ends up sequestered and degraded in the liver or removed by the kidneys. Since the common culprit in this scenario often is the monoclonal, the acronym HAMA, for *human anti-mouse antibody*, is given to the response.

In an attempt to overcome the HAMA problem, "humanized" mouse monoclonals were designed where large portions of the murine antibody are substituted for their human counterparts. For instance, replacing a mouse Fc portion with the corresponding human ones can significantly decrease the immune response against such conjugates. Replacing everything but the hypervariable regions which code for antigen binding has been accomplished, too. Unfortunately, regardless of how much "humanization" is done, the remaining murine part still has the potential of causing immunological reactions. The advent of recombinant antibodies of completely human origin essentially has overcome the problem of immune system response to the antibody component. However, there still is the potential for generating an immune response from the toxic part of the immunoconjugate, but this is unavoidable unless immune modulators are administered to reduce the overall immune system responsiveness.

Modification of immunotoxin conjugates with synthetic polymers has been used to mask the complex from the host immune response. Particularly, polyethylene glycol (PEG; Chapter 25, Section 1 and Chapter 18) has been found to be quite successful in reducing or eliminating immune system reactions (Roffler and Tseng, 1994). Modification of an antibody conjugate with 2–4 PEG molecules increases the serum half-life and improves tumor localization of the targeted reagent.

Other innovations in preparing targeted conjugates for cancer utilize recombinant DNA techniques to create antibody molecules that are entirely of human origin (Huse *et al*., 1989; Orlandi *et al*., 1989; Sastry *et al*., 1989; Hudson and Souriqu, 2007). A completely human antibody molecule eliminates the immunological problems associated with mouse monoclonals. Intact antibodies, Fab fragments, small Fv fragments held together by synthetically designed amino acid segments, and short peptides representing the antigen binding site have all been developed by recombinant means. Although frequently the word "antibody" is used to describe these engineered proteins, many of the molecules are far removed from the traditional picture of an antibody molecule. The terms "single chain antibody" or "single chain Fv protein" are commonly used and more closely describe these new targeting molecules.

With the great diversity of targeted toxic agents being developed for cancer therapy, it would be difficult to characterize this section strictly as antibody conjugation. While many,

if not most, studies utilize monoclonal antibodies of one form or another as the biospecific-targeting component, the complete picture involves the crosslinking of a wide variety of molecules together to create the final conjugate. This section presents some of the most common methods of immunotoxin preparation. For the preparation of other unique targeted toxin conjugates, the methods found throughout this book for linking one particular functional group to another can be followed with an excellent probability for success.

1. Properties and Use of Immunotoxin Conjugates

Conjugates of monoclonal antibodies and protein toxins are undergoing extensive research for their usefulness in the treatment of cancer. Toxins of many different types can be used to create effective immunotoxin conjugates, including the proteins ricin from castor beans (*Ricinus communis*), abrin from *Abrus precatorius*, modeccin, gelonin from *Gelonium multiflorum* seeds, diphtheria toxin produced by *Corynebacterium diphtheriae*, pokeweed antiviral proteins (PAPs; three types: PAP, PAP-II, and PAP-S) from *Phytolacca americana* seeds, cobra venom factor (CVF), *Pseudomonas* exotoxin, restrictocin from *Aspergillus Restrictus*, momordin from *Momordica Charantia* seeds, saporin from *Saponaria officinalis* seeds, as well as other ribosome-inactivating proteins (RIPs).

By far the most popular choices for the toxin component of immunotoxins are ricin, abrin, modeccin, and diphtheria toxin. The three plant toxins have lectin binding activity toward terminal β-galactosyl residues and they can be inhibited by the presence of simple sugars like galactose and lactose. The toxin proteins bind to cell-surface polysaccharide receptors with high affinity (k_a in the range of 10^7–10^8). Ricin, abrin, and modeccin consist of two subunits with remarkably similar structures and activities. The intact proteins have molecular weights (MW) of approximately 63–65,000 with each subunit of about equal size. The subunits are joined by disulfide linkages that are important reversible bonds in the mechanism of cytotoxicity. The A chain is called the effectomer and possesses ribosomal-inactivating properties. The B chain contains the carbohydrate binding site and it is termed the haptomer. While the intact toxin molecules have potent cytotoxic effects on cells, they exhibit no ribosomal-inactivating activity on ribosomes in a cell-free system. By contrast, reduction of the toxins with a disulfide reducing agent creates the opposite effects. Reduced, dissociated toxin subunits inhibit ribosomal activity in cell-free systems, but they have no affect on intact cells.

The reason for these properties is due to the toxins' mode of action. Toxin molecules bind through saccharide-recognition sites on the B chain to particular β-galactosyl-containing glycoprotein or glycolipid components on the surface of cell membranes. In animals sensitive to these toxins, the necessary polysaccharide ligands are present in large quantities on virtually all cell types (Cumber *et al*., 1985). Upon binding of the dimer to the cell, the A chain enters the cell either by active transport into endocytic vesicles or through some mechanism of its own. Once inside the cell membrane, the A chain enters the cytoplasmic space, binding to and enzymatically inactivating the 60S subunit of ribosomes (Olsnes and Pihl, 1976, 1982). The result is cessation of protein synthesis and eventual cell death. Because the A chain's action is through enzymatic means, as little as one active toxin molecule is enough to seriously disrupt protein synthesis operations and probably sufficient to kill a target cell (Eiklid *et al*., 1980). The turnover rate of one A-chain molecule is about 1,500 ribosomes inactivated per minute (Olsnes, 1978).

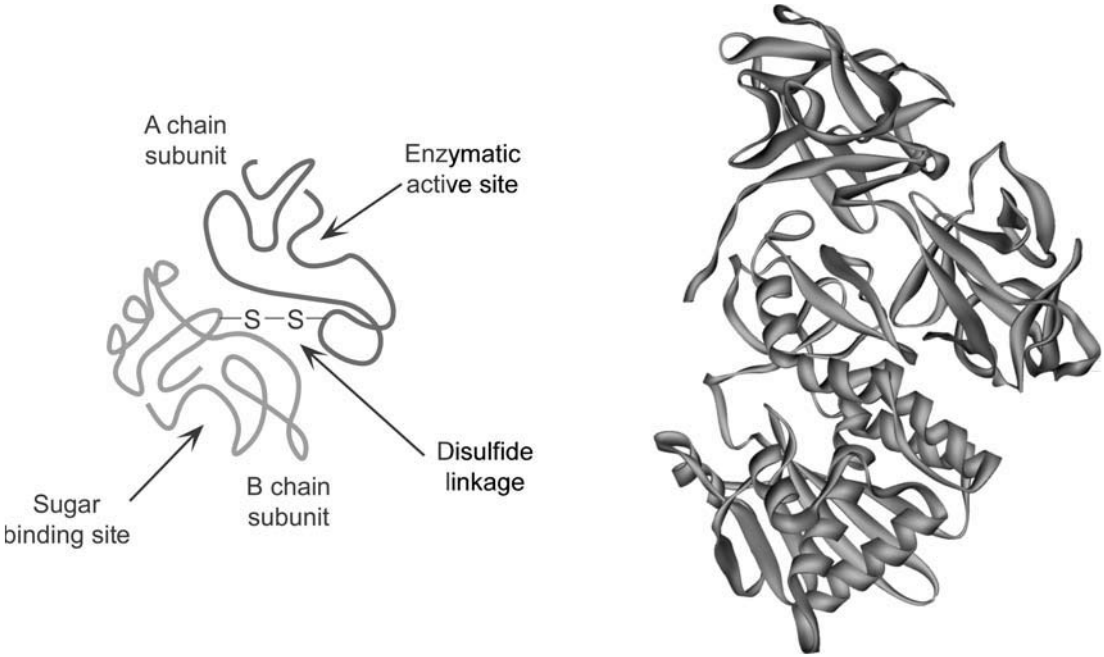

Figure 21.2 Conceptualized construction of an A–B subunit protein toxin (left). The B chain contains a binding region for docking onto cell surfaces, while the A chain contains a catalytic site that produces cytotoxic affects intracellularly. The two subunits are joined by a disulfide bond that is reductively cleaved at the cellular level to allow the A subunit to affect cell death. A molecular model of ricin is on the right.

Diphtheria toxin also is a two-subunit protein, but it is initially synthesized by certain strains of *Corynebacterium diphtheriae* as a single polypeptide chain of MW 63,000. Proteolytic processing results in the formation of a "nicked toxin" which is enzymatically inactive, but consists of two subunits bonded together by an interchain cystine disulfide. Upon reduction of the disulfide, the A chain (MW 24,000) is released and manifests enzymatic activity toward ribosomal proteins (Sandvig and Olsnes, 1981; Collier and Cole, 1969). Its mode of action is different than the plant toxins. The A-chain fragment of diphtheria toxin catalyzes the ADP-ribosylation of eukaryotic aminoacyl-transferase II (EF2) using NAD^+ (Honjo *et al.*, 1968; Gill *et al.*, 1969). The B chain, by contrast, possesses no enzymatic activity, but evidence points to the fact that a binding site on it recognizes certain cell-surface receptors. As in the action of ricin, abrin, and modeccin, the B chain of diphtheria toxin is necessary for cytotoxicity (Colombatti *et al.*, 1986). There also is a role for the C-terminus cysteine residue of the B chain in cell penetration (Dell'Arciprete *et al.*, 1988).

Figure 21.2 illustrates the basic structure of these common two-subunit toxins, showing schematically their major characteristics. The molecular model of ricin is from Rutenber *et al.* (1991), RSCB structure No. 2aai.

Due to the extraordinary toxicity of intact ribosome-inactivating toxins like ricin, abrin, and modeccin, purification and handling of these proteins must be done with extreme care. Even dust from crude seed powders or lyophilized proteins should be considered dangerous. During

the height of the cold war days, a Soviet KGB agent killed a man from the West by injecting at most only milligram quantities of ricin into his leg using a modified umbrella tip. There even have been instances of worker deaths at companies that routinely purify these proteins. For this reason, all handling operations of intact toxin dimers and purified subunits should be done in fume or laminar-flow hoods. Avoid, also, the use of laboratory tools that could lead to puncture wounds causing contaminating toxin injection.

Some potentially cytotoxic proteins contain only a single polypeptide chain, such as gelonin and PAPs. Such toxins manifest similar enzymatic ribosome-inactivating properties as the multi-subunit proteins like ricin, but do not possess the cell-recognition capacity that the B-chain subunit contains. The result is the inability of these toxins to bind or affect intact cells. However, they do maintain the typical ribosome-inactivating properties in a cell-free system that the A chain of two-subunit toxins possess. If these proteins are conjugated with a cell-targeting agent, such as the B chain of ricin or a specific antibody that recognizes cell-surface epitopes, full cytotoxicity results.

Gelonin and PAPs are much more convenient to work with than ricin and the other two-subunit toxins. Most importantly, they are relatively nontoxic to cells unless conjugated with something that can facilitate cell binding and internalization (Stirpe *et al*., 1980; Irvin, 1983). The pI of these toxins is in the basic range, and they each have a MW of about 30,000 (Barbieri and Stirpe, 1982). These single-subunit proteins are very stable, especially to purification techniques, but also to most modification and crosslinking steps associated with preparing immunotoxins. Studies have shown (Lambert *et al*., 1985) that modification of gelonin or PAPs can be done with 2-iminothiolane (Chapter 1, Section 4.1) to create sulfhydryl groups without loss of activity. Previous studies, however, have determined that the use of SPDP to modify gelonin resulted in a 90 percent inactivation (Thorpe *et al*., 1981). The difference in these results may be due to the retention of positive charge characteristics on the modified amine when using Traut's reagent, but neutralization of that charge when using SPDP.

Immunotoxin conjugates consist of an antibody covalently crosslinked to a toxin molecule in a way that maintains the unique properties of both proteins. The antibody component consists of a monoclonal having specificity for an antigenic determinant on the surface of a particular cell type. Most often, the targeted cells are tumors that express a unique cell-surface marker which can be recognized by the monoclonal. The role of the antibody, therefore, is to function as a passive taxi, carrying the toxin component to the targeted cells. Once at the tumor location, the toxin component effects its intended ribosome-inhibiting action, ultimately causing cell death and tumor destruction.

Since immunotoxin conjugates are destined to be used *in vivo*, their preparation involves more critical consideration of crosslinking methods than most of the other conjugation protocols described in this book. The following sections discuss the problems associated with toxin conjugates and the main crosslinking methods for preparing them.

2. Preparation of Immunotoxin Conjugates

It has become apparent that the method of crosslinking can dramatically affect the activity of an immunotoxin *in vivo*. This is true not only with regard to possible direct blocking by the crosslinker of the enzymatic active site which is responsible for inactivation of ribosomes, but the chemistry of conjugation also is an important factor in proper binding and entry of the

conjugate into the cell. Preparation of the conjugate should maintain the antigen binding character of the attached antibody and at the same time not block the ribosome-inactivating activity of the toxin component.

Studies have been done to investigate the importance of using a cleavable linker between the antibody and the toxin. This configuration in the immunoconjugate would mimic the natural state of two-subunit toxins like ricin that are held together by disulfide bonds. There is evidence that disulfide reduction and cleavage of the A chain from the B chain is necessary for cytotoxicity in native toxins (Olsnes, 1978). There is similar evidence that the creation of cytotoxic immunotoxins using only A-chain subunits requires that the conjugation be done with a monoclonal using a crosslinker that possesses a disulfide bond in its cross-bridge or creates a disulfide linkage upon coupling (Masuho *et al*., 1982). Using disulfide-cleavable crosslinkers in the preparation of immunotoxins results in the antibody taking on the role of the B chain in recognizing and binding to antigenic determinants on the surface of cells. After binding, some mechanism internalizes the conjugate where the two components then are separated by disulfide reduction. The A-chain subunit is then freed to enter the cytoplasmic space where enzymatic degradation of the ribosomal proteins occurs.

Other investigators, however, have demonstrated that conjugations of antibody with intact, two-subunit toxins can be done using non-cleavable crosslinkers such as NHS ester–maleimide heterobifunctionals (Chapter 5, Section 1) (Myers *et al*., 1989). Presumably, the toxin is still able to release the A chain after the antibody has bound to the cell, since the conjugation process does not permanently attach the two toxin subunits together—only the toxin to the antibody.

Thus, two main strategies can be used in making immunotoxin conjugates (Figure 21.3). In the most often used method, the isolated A chain of two-subunit toxins (or the intact polypeptide of single-subunit toxins like gelonin) is conjugated to a monoclonal using a crosslinker that can introduce a disulfide bond. When using only purified A chain, it is common (but not absolutely required) to couple through the sulfhydryl that is freed during A–B chain cleavage by disulfide reduction. The single-chain toxins like gelonin, however, have no free sulfhydryls, so a thiolating agent such as 2-iminothiolane (Chapter 1, Section 4.1) may be used to create them (Lambert *et al*., 1985).

When using ricin A chains, it has been found that chemical deglycosylation of the subunit prevents its nonspecific binding to receptors for mannose on certain cells of the reticuloendothelial system (Vitetta and Thorpe, 1985; Ghetie *et al*., 1988, 1991; O'Hare *et al*., 1988). Thus, immunotoxin conjugates consisting of deglycosylated ricin A chain (dgA) have been shown to survive longer *in vivo* and are more efficient at reaching their intended target cells. In addition, if the antibody component does not contain Fc region, but consists of only $F(ab')_2$, Fab′, Fab, or smaller Fv fragments, then nonspecific binding of the immunotoxin *in vivo* will be reduced to a minimum. One study found that constructing immunotoxin conjugates with molar ratios of two dgA per antibody molecule resulted in a 7-fold increase in cytotoxicity over a 1:1 conjugate ratio (Ghetie *et al*., 1993).

A-chain immunotoxins, however, may not be quite as cytotoxic as conjugates formed from intact toxin molecules (Manske *et al*., 1989). In an alternative approach to A chain use, the intact toxin of two-subunit proteins is directly conjugated to a monoclonal without isolation of the A chain. Conjugation of an antibody with intact A–B chain toxins can be done without a cleavable linker, as long as the A chain can still separate from the B chain once it is internalized. Therefore, it is important to avoid intramolecular crosslinking during the conjugation process which can prevent release of the A–B complex. In addition, since the B chain

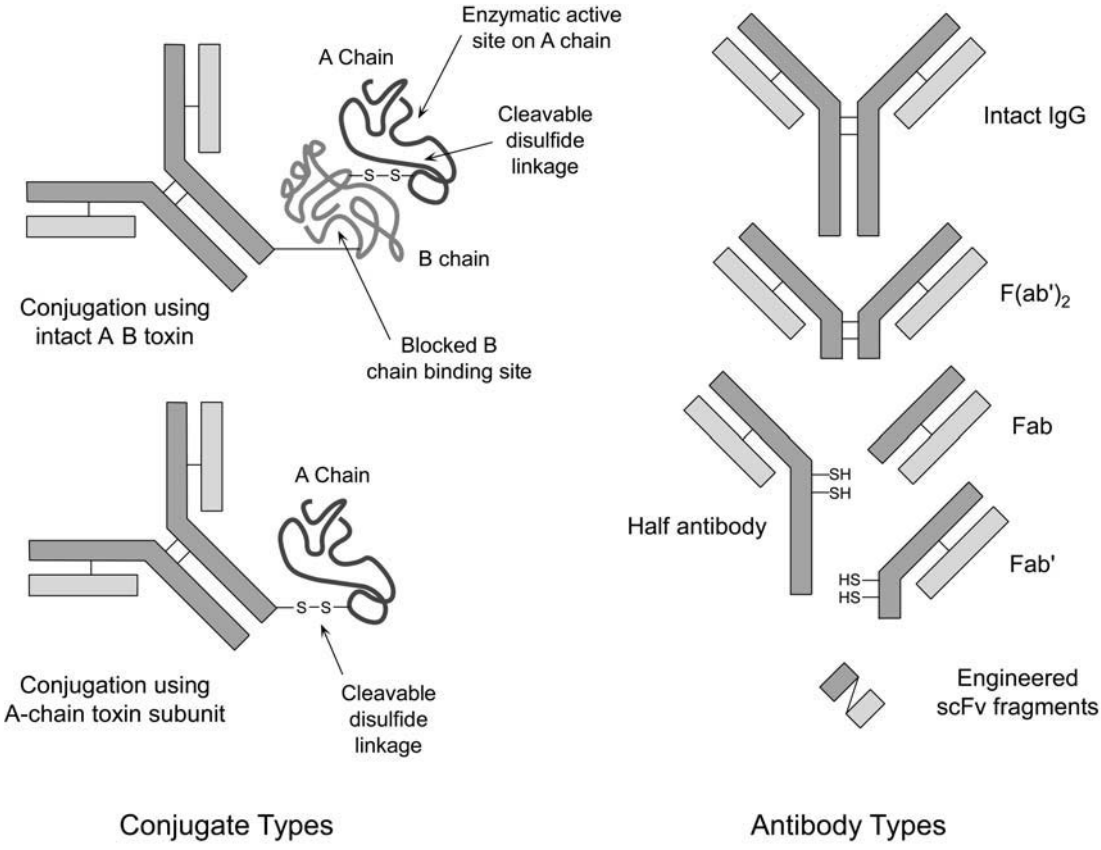

Figure 21.3 The strategies involved in creating an immunotoxin conjugate are numerous. Intact antibody molecules or enzymatic fragments may be used as the targeting component. Even small recombinant Fv fragments that are genetically engineered to limit host immune response can be employed. Conjugates can include two-subunit toxins or purified A-chain components. If intact toxins are used, the B-chain binding site must be blocked to prevent nonspecific cell death. If A-chain subunits are used, to induce cytotoxic effects in the conjugate the crosslinking agent must generate a disulfide bond that can allow toxin release.

possesses a recognition site for most cell surfaces, it still has the ability to nonselectively bind and kill non-tumor cells. To maintain antibody specificity in intact toxin conjugates toward only one cell type (and thus prevent nonspecific cell death), all cell binding capability within the toxin itself must be removed. Fortunately, a large proportion of the binding sites on the B chains usually are blocked during the conjugation process, and the galactose binding potential is significantly impaired. Further purification to remove conjugates that have remaining galactose binding potential can be done on an acid-treated agarose chromatography column (which contains galactose residues) or on a column of asialofetuin bound to agarose (Cumber *et al.*, 1985). Conjugate fractions which do not bind to both affinity gels contain no nonspecific binding potential toward non-targeted cells.

More elaborate methods of blocking or eliminating the B-chain galactose binding site also can be done to prevent nonspecific cytotoxicity. For instance, the crosslinking agent may have a

lactose molecule designed into it that can block B-chain activity. The lactose portion possesses natural affinity for the B-chain binding site, and thus it occupies that area while a nearby reactive group covalently attaches the crosslinker to neighboring functional groups on the protein.

Moroney *et al.* (1987) used this approach in creating a ricin conjugate. Lactose was modified at its reducing end with cystamine via reductive amination (Chapter 2, Section 5.3). The cystamine was reduced with dithiothreitol (DTT) and immobilized on an affinity gel which was activated with a pyridyl dithiol group (Chapter 2, Section 2.6). The coupled lactose then was modified with a chlorotriazine derivative through the secondary amine created by the reductive amination process. Next, the ricin molecule was immobilized by the additional reactive group on the chlorotriazine ring. Since this reactive group was immediately adjacent to the lactose residue, the ricin bound the sugar at its B-chain binding site and it was covalently coupled through a nearby amine. This process effectively blocked and eliminated all nonspecific binding potential in the ricin dimer. After removal of the blocked ricin from the support by DTT, the free sulfhydryl group on the protein was conjugated to an SMCC-modified antibody, forming the final conjugate (Figure 21.4). Although this process worked in preparing an effective immunotoxin conjugate, most conjugation schemes are less elaborate.

Regardless of their method of preparation, the required and ideal characteristics of immunotoxin conjugates can be summarized in the following points:

1. The conjugation process must leave the antigen binding sites on the antibody component free to interact with its intended target. Crosslinker modification or blockage of these binding sites by the attached toxin must be kept to a minimum.
2. The toxin component of the conjugate must be able to elicit cytotoxicity by ribosomal damage as it could in its native state. This means that the cell penetration and enzymatic properties of the toxin remain unaltered, although an antibody molecule is conjugated to it.
3. The activity of toxin binding to cells through the B chain must be eliminated in the conjugate to prevent nonspecific binding and nonselective cell death. This may be accomplished by using only the A-chain subunit or by blocking the B-chain binding site in the intact toxin conjugate.
4. Avoid covalently linking the A and B chains together during the crosslinking process. This can be done by using heterobifunctional crosslinkers that are more controllable in their reactivity than homobifunctional reagents.
5. The crosslinking process must minimize polymerization of either the antibody or the toxin. Low molecular weight, 1:1 or 1:2 conjugates of antibody-to-toxin are best.
6. The crosslinker used to form the bond between the antibody and toxin must be able to survive *in vivo* and not be cleaved by enzymatic or reductive means before reaching the targeted cells.
7. The conjugate must reach its intended target without being intercepted, bound and destroyed by the host immune system.
8. Administration of the immunotoxin should result in cell death and complete elimination of all target cells.

The last two points are the most difficult to realize. The following methods describing the conjugation strategies used to prepare immunotoxins work well in creating complexes containing active antibody and toxin. The majority of research today is not so much concerned with further optimization of the crosslinking process, but primarily is directed at overcoming the

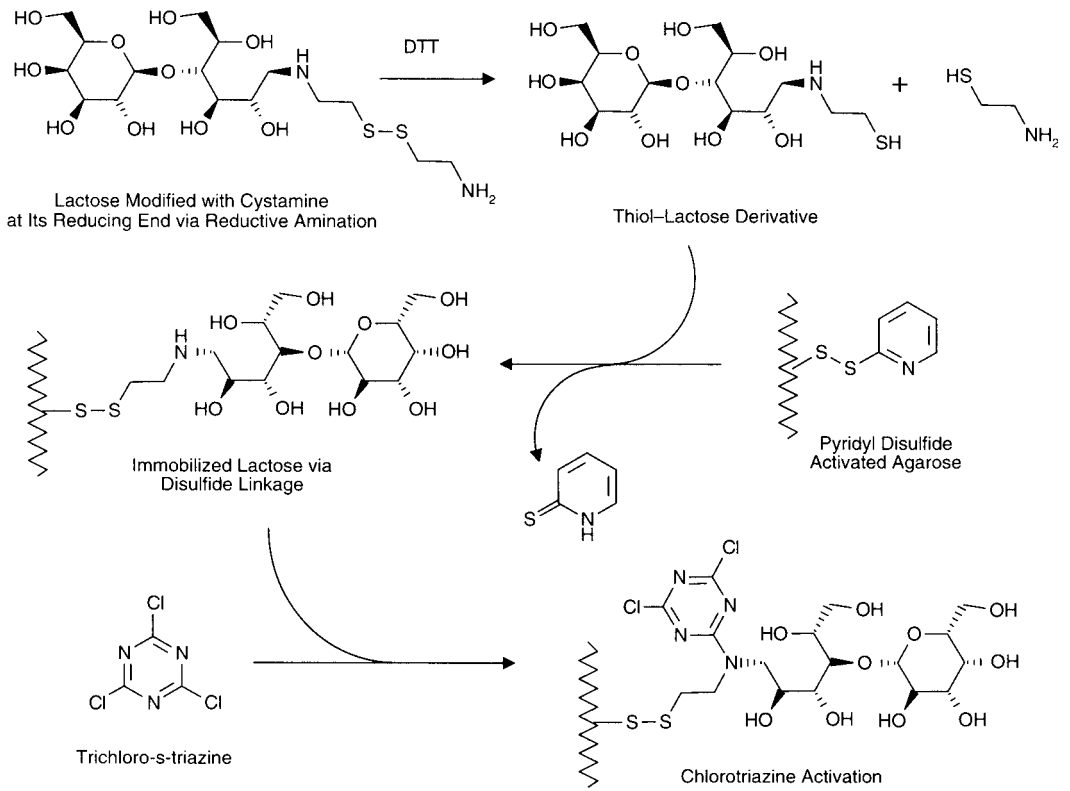

Figure 21.4 In an elaborate strategy to block the B-chain binding site in the construction of immunotoxins using intact A–B subunit toxins, cystamine was first coupled to the reducing end of lactose by reductive amination. DTT was then used to reduce the cystamine disulfide group, revealing the free thiol. A pyridyl disulfide-activated agarose gel then was used to couple the lactose derivative through its sulfhydryl. Next, trichloro-s-triazine was reacted with the support to modify the secondary amine on the cystamine component, forming a reactive gel. Finally, addition of an intact toxin to the affinity support caused binding with the lactose group at the B-chain binding site. Since the B chain was now in proximity to the chlorotriazine ring, covalent coupling occurred with available amines on the protein toxin, thus permanently blocking the binding site. After removal of the modified toxin from the gel using a disulfide reducing agent, the free thiol of the cystamine group was used to conjugate with an SMCC-activated immunoglobulin.

host immune system and making the conjugates more effective in accomplishing complete targeted tumor destruction.

2.1. Preparation of Immunotoxin Conjugates via Disulfide Exchange Reactions

Since the cytotoxic potential of most common toxins relies on their subunit disulfide cleavability with subsequent release of associated A chains, most successful conjugation techniques for preparing immunotoxins involve the use of disulfide exchange reactions. Heterobifunctional crosslinking agents containing an amine-reactive group at one end and a disulfide bond with a

good leaving group on the other end are common choices for making these conjugates (Chapter 5, Section 1). The leaving group on the disulfide portion of the crosslinker permits efficient disulfide interchange with a free sulfhydryl on the antibody or toxin. The resultant covalent bond thus is a cleavable disulfide which mimics the native cleavability inherent in the toxin dimer.

Pyridyl Disulfide Reagents

The most common reactive group for initiating disulfide interchange reactions is a pyridyl disulfide. Attack of a nucleophilic thiolate anion dissociates the pyridine-2-thione leaving group from this group, forming a new disulfide bond with the incoming sulfhydryl compound. Several crosslinking reagents containing these groups are popular choices for producing antibody–toxin conjugates.

SPDP

SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate, is by far the most popular heterobifunctional crosslinking agent used for immunotoxin conjugation (Chapter 5, Section 1.1). The activated NHS ester end of SPDP reacts with amine groups in one of the two proteins to form an amide linkage. The 2-pyridyldithiol group at the other end reacts with sulfhydryl groups in the other protein to form a disulfide linkage (Carlsson *et al.*, 1978). The result is a crosslinked antibody–toxin conjugate containing cleavable disulfide bonds that can emulate the activity of native two-subunit toxin molecules.

LC-SPDP (Chapter 5, Section 1.1) is an analog of SPDP containing a hexanoate spacer arm within its internal cross-bridge. The increased length of the extended crosslinker is important in some conjugations to avoid steric problems associated with closely linked macromolecules. However, for the preparation of immunotoxins, no advantages were observed for LC-SPDP over SPDP (Singh *et al.*, 1993).

SPDP is also useful in creating sulfhydryls in one of the two proteins being conjugated (Chapter 1, Section 4.1). Once modified with SPDP, the protein can be treated with DTT (or another disulfide reducing agent) to release the pyridine-2-thione leaving group and form the free sulfhydryl. The terminal —SH group then can be used to conjugate with any crosslinking agents containing sulfhydryl-reactive groups, such as maleimide or iodoacetyl (for covalent conjugation) or 2-pyridyldithiol groups (for reversible conjugation).

In the preparation of immunotoxins, some procedures call for the modification of the antibody with SPDP to introduce reactive thiols (Cumber *et al.*, 1985). The NHS ester end of the crosslinker is reacted at slightly alkaline pH with the primary amines on the antibody. After removal of excess reagent by gel filtration, the pyridyl disulfide groups are reduced by DTT. The reductant causes the removal of pyridine-2-thione groups and the creation of sulfhydryl groups on the immunoglobulin. The reason the antibody is thiolated in this manner and not the toxin is to avoid exposing the intact toxin to reducing conditions that could disassociate the A and B subunits. To activate the toxin, SPDP again can be used to modify the intact A–B component. After purification of the modified toxin from excess crosslinker, the SPDP–toxin is mixed with the thiolated antibody to effect the final conjugate (Figure 21.5).

This multi-step crosslinking method employing SPDP on both molecules has been used to prepare a number of immunotoxin conjugates (Edwards *et al.*, 1982; Thorpe *et al.*, 1982; Colombatti *et al.*, 1983; Wiels *et al.*, 1984; Vogel, 1987; Reiter and Fishelson, 1989). While

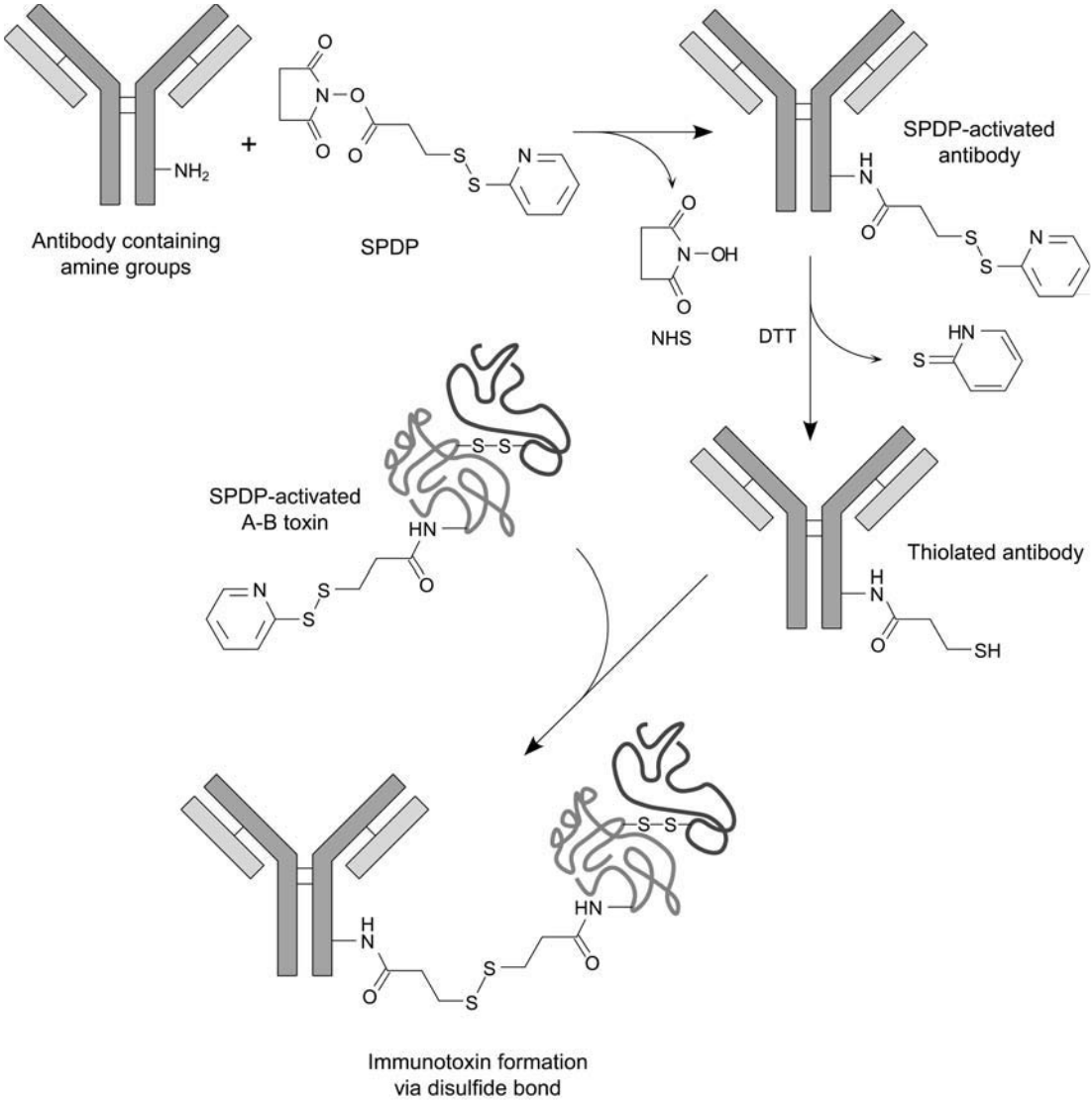

Figure 21.5 SPDP can be used to modify both an antibody and a toxin molecule for conjugation purposes. In this case, the antibody is thiolated to contain a sulfhydryl group by modification with SPDP followed by reduction with DTT. A toxin molecule is then activated with SPDP and reacted with the thiolated antibody to effect the final conjugate through a disulfide bond.

this method has worked well for many different toxins, its main potential disadvantage is exposure of the antibody to reducing conditions that potentially could cleave the disulfide bonds holding its heavy and light chains together. Alternative methods using SPDP in a nonreducing environment may result in better conjugates.

For instance, if toxin A chain–antibody conjugates are to be prepared, the antibody can be similarly activated with SPDP, but in this case not treated with reductant. After removal of

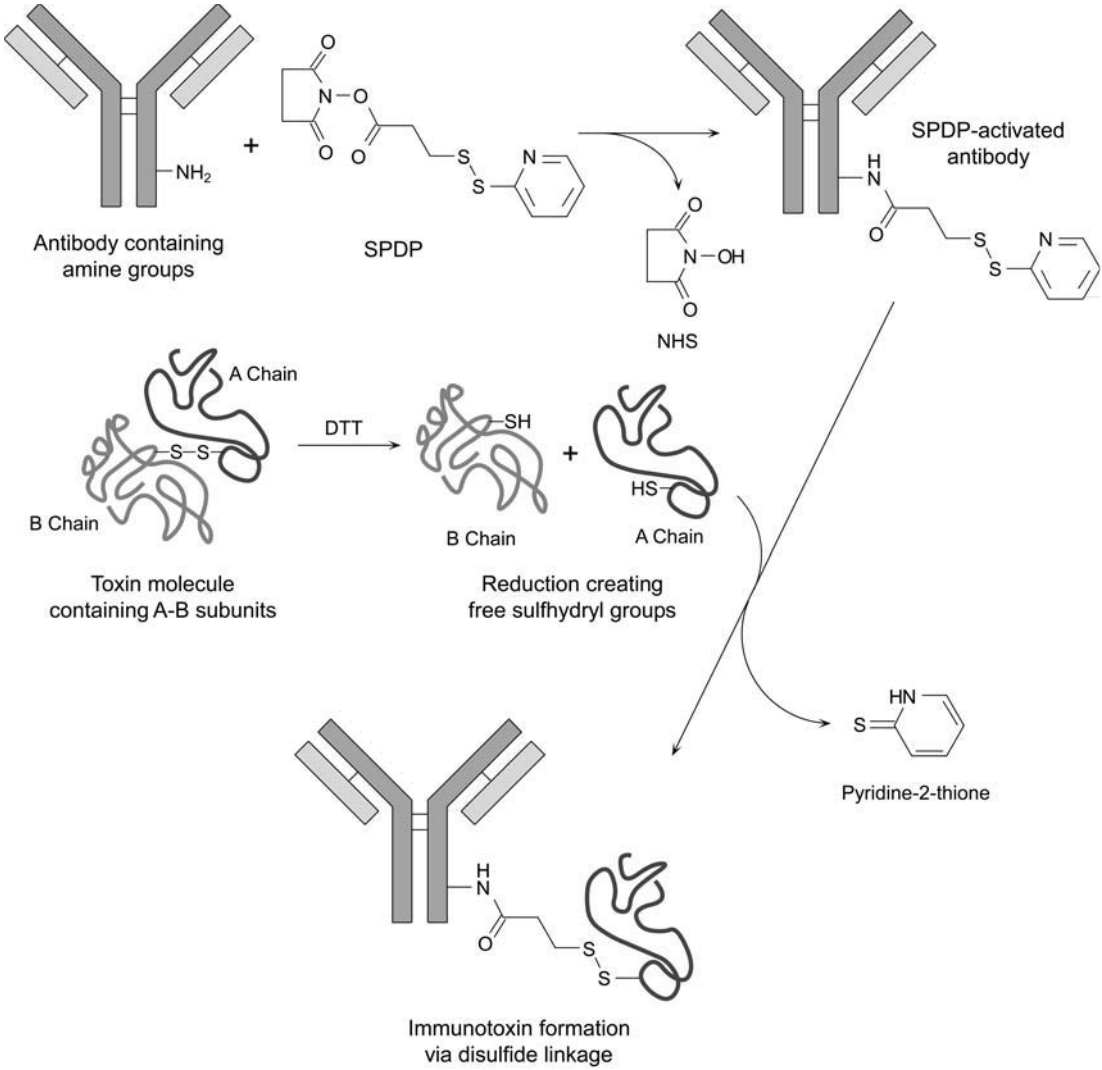

Figure 21.6 SPDP can be used to activate an antibody molecule through its available amine groups to form a sulfhydryl-reactive derivative. Toxin molecules containing disulfide-linked A–B chains may be reduced with DTT to isolate the A-chain component containing a free thiol. The SPDP-activated antibody is then mixed with the reduced A chain to effect the final conjugate by disulfide bond formation.

excess crosslinker, the activated antibody can be directly mixed with isolated A chain to create the conjugate (Figure 21.6). This procedure makes use of the indigenous sulfhydryl residues produced during reductive separation of the A and B chains and therefore does not require crosslinker thiolation of one of the proteins.

Another way of utilizing SPDP is to again activate the antibody to create the pyridyl disulfide derivative, but this time thiolate the toxin component using 2-iminothiolane (Chapter 1,

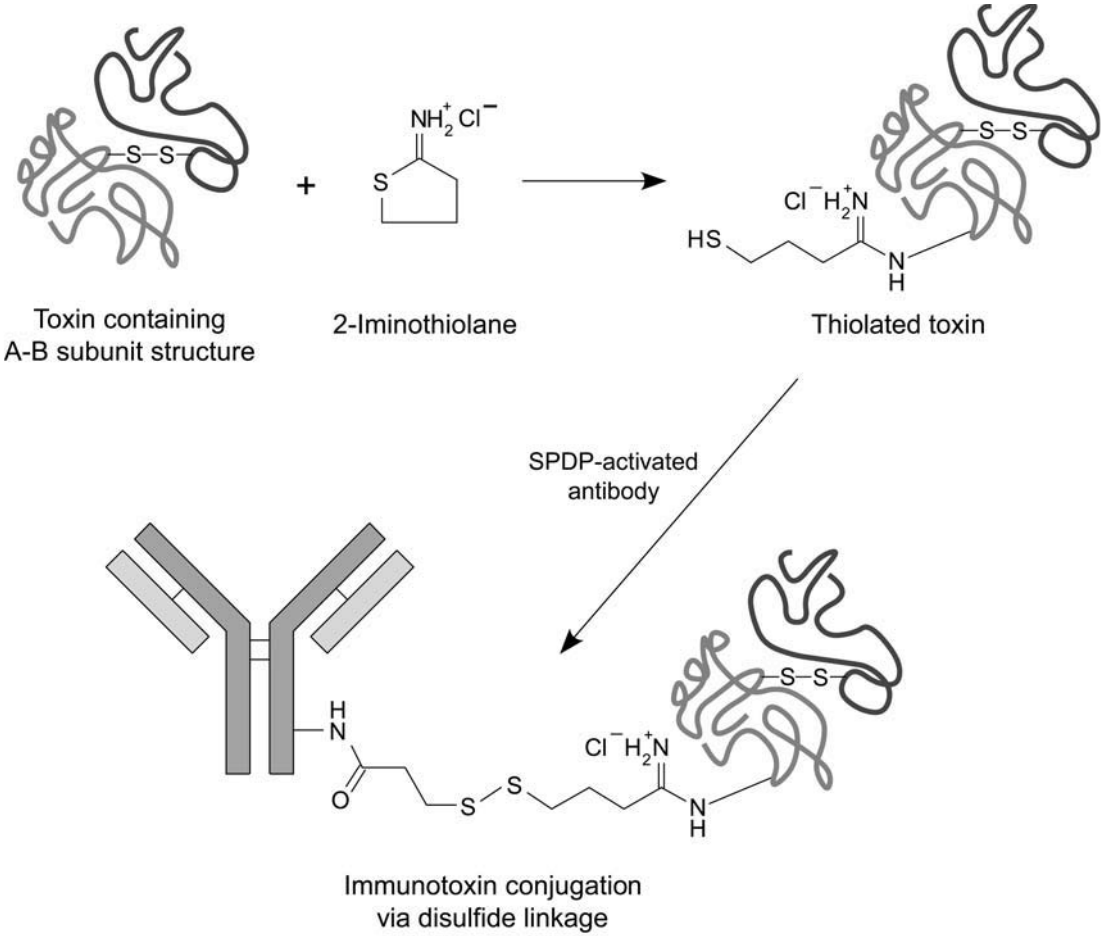

Figure 21.7 An intact A–B subunit toxin molecule may be activated with 2-iminothiolane with good retention of cytotoxic activity. The thiolated toxin then may be conjugated with SPDP-activated antibody to generate the immunotoxin conjugate through a disulfide bond.

Section 4.1). 2-Iminothiolane reacts with primary amines in a ring-opening reaction that creates a terminal sulfhydryl group without reduction. Intact A–B toxins and toxins containing only one subunit, like gelonin, PAPs, and Pseudomonas toxin A, can be coupled to antibodies using this procedure (Lambert *et al*., 1985; Bjorn *et al*., 1986; Scott *et al*., 1987; Lambert *et al*., 1988; Ozawa *et al*., 1989). Mixing the SPDP-activated antibody with the thiolated toxin effects the final conjugation (Figure 21.7).

SPDP also can be used to conjugate other targeting molecules to toxins, such as transferrin, epidermal growth factor, α_2-macroglobulin, and human chorionic gonadotropin (Fizgerald *et al*., 1980; Helenius *et al*., 1980; Keen *et al*., 1982; Oeltmann, 1985). To create these conjugates, one of the two components must be activated with SPDP to generate the sulfhydryl-reactive pyridyl disulfide groups, while the other component must be modified to contain the —SH functionality. Mixing these modified molecules together forms the toxin conjugate.

The following methods are generalized to provide an overview of how SPDP can be used in these conjugation techniques. The appropriate optimization for a particular toxin conjugate should be done.

Caution! Toxins are highly toxic even in very low amounts. Handle all toxin molecules and their isolated subunits with extreme care.

Protocol for Thiolation of Antibody with SPDP and Conjugation to an SPDP-Activated Toxin

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

Note: In this protocol, for every mg of toxin employed, 2.5 mg of antibody is required to obtain the correct molar ratios in the final conjugate.

Activation of Toxin with SPDP

1. Dissolve the toxin to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 10 mg/ml. Some protocols use as an SPDP reaction buffer, 50 mM sodium borate, 0.3 M NaCl, 0.5 percent *n*-butanol, pH 9.0. Both buffer systems work well for the NHS ester modification reaction, although the pH 9 buffer is at the higher end of effective derivatization with active esters, since the hydrolysis rate is dramatically increased at this level of alkalinity.
2. Dissolve SPDP (Thermo Fisher) in DMF at a concentration of 3 mg/ml. Add 20 µl of this solution to each ml of the toxin solution. Gently mix to effect dissolution. Retain the SPDP stock solution for use in the antibody modification step, below.
3. React for 30 minutes at room temperature.
4. Purify the SPDP-activated toxin from excess reagents and reaction by-products by gel filtration using a desalting resin. For the chromatography use 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, containing 10 mM EDTA.
5. Concentrate the toxin to 10 mg/ml using a centrifugal concentrator with a MW cutoff of 10,000. Retain this solution for the conjugation reaction.

Thiolation of Antibody with SPDP

1. Dissolve the antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration 10 mg/ml. *Note*: some protocols use the borate buffer system described in step A. Use 2.5 mg of antibody per mg of toxin to be conjugated.
2. Dissolve SPDP in DMF at a concentration of 3 mg/ml. Add 24 µl of this solution to each ml of the antibody solution with gentle mixing to effect complete dissolution.
3. React for 30 minutes at room temperature.
4. Remove excess crosslinker by gel filtration using a desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5. The buffer should be degassed under vacuum and nitrogen bubbled through it to remove oxygen. The presence of EDTA stabilizes the free sulfhydryls formed in the following steps against metal-catalyzed oxidation.
5. Concentrate the fractions containing protein from the gel filtration step to 10 mg/ml using a centrifugal concentrator (MW cutoff of 10,000).

6. To reduce the pyridyl dithiol groups and create reactive sulfhydryls, dissolve DTT in water at a concentration of 17.2 mg/ml and immediately add 500 µl of this solution to each ml of concentrated antibody solution. Mix to dissolve and react for 30 minutes at room temperature.
7. Remove excess DTT by gel filtration using the same buffer as in step 4. Pool the fractions containing protein and concentrate to 10 mg/ml.

Conjugation of SPDP-Activated Toxin with Thiolated Antibody

1. Immediately mix the concentrated, thiolated antibody solution from part B with the SPDP-activated toxin from part A.
2. React for 18 hours at room temperature to form the final conjugate. Isolation of the ideal 1:1 or 1:2 antibody–toxin conjugate can be done through gel filtration separation using a column of Sephacryl S-300 or the equivalent.

Protocol for Activation of Antibody with SPDP and Conjugation to a Toxin A Chain

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

Since the A chain of toxin molecules contains a free sulfhydryl group, there is no need in this conjugation strategy to thiolate one of the molecules. The following protocol calls for 1.73 mg of antibody per mg of toxin A chain to produce a conjugate possessing the correct molar ratio of components. Best results for creating a highly cytotoxic immunotoxin will be obtained if deglycosylated ricin A chain is used.

1. Dissolve the antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration 10 mg/ml.
2. Dissolve SPDP (Thermo Fisher) in DMF at a concentration of 3.0 mg/ml. Add 30 µl of this solution to each ml of the antibody solution with gentle mixing to effect complete dissolution.
3. React for 30 minutes at room temperature.
4. Remove excess crosslinker by gel filtration using a desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5.
5. Concentrate the fractions containing SPDP-activated antibody from the gel filtration step to 10 mg/ml using a centrifugal concentrator (MW cutoff of 10,000).
6. Mix the activated antibody solution with a solution of deglycosylated toxin A chain (dgA) dissolved in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, containing 10 mM EDTA. The ratio of mixing should equal 1.73 mg of antibody per mg of A chain or 580 µl of A-chain solution at 10 mg/ml per ml of activated antibody solution at 10 mg/ml. The A-chain solution must not contain any reducing agents left over from the disassociation of the toxin subunits during the A subunit isolation. Reductants will compete for the SPDP activation sites on the antibody molecule.
7. React for 18 hours at room temperature. Isolation of the 1:1 or 1:2 antibody–toxin conjugate can be done through a gel filtration separation using a column of Sephacryl S-200.

Isolation of conjugates containing molar ratios of 1:2 antibody:dgA have resulted in greater cytotoxicity behavior *in vivo* (Ghetie *et al*., 1993).

Protocol for the Conjugation of SPDP-activated Antibodies with 2-Iminothiolane-Modified Toxins

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

A third option for immunotoxin preparation is to again activate the antibody with SPDP, while this time thiolating a single-chain toxin molecule to conjugate with it. This method works especially well using 2-iminothiolane (Chapter 1, Section 4.1) to create sulfhydryls on gelonin or PAPs. Gelonin is a single-polypeptide toxin containing no free sulfhydryls. A number of options are available for thiolation, however the use of SPDP to add sulfhydryl groups inactivates the toxin, while 2-iminothiolane preserves its activity, perhaps by maintaining the positive charge on the amines that are being modified (Lambert *et al*., 1985).

Activation of Antibody with SPDP

1. Dissolve the antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 10 mg/ml.
2. Dissolve SPDP (Thermo Fisher) in DMF at a concentration of 3.0 mg/ml. Add 30 µl of this solution to each ml of the antibody solution with gentle mixing to effect dissolution.
3. React for 30 minutes at room temperature.
4. Remove excess crosslinker by gel filtration using a desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5.
5. Concentrate the fractions containing SPDP-activated antibody from the gel filtration step to 10 mg/ml using a centrifugal concentrator (MW cutoff of 10,000).

Thiolation of Gelonin (or Other Single-Polypeptide Toxins) with 2-Iminothiolane (Traut's Reagent)

1. Dissolve gelonin at a concentration of 10 mg/ml in 50 mM triethanolamine hydrochloride, pH 8.0, containing 10 mM EDTA. The buffer should be degassed under vacuum and bubbled with nitrogen to remove oxygen that may cause sulfhydryl oxidation after thiolation.
2. Dissolve 2-iminothiolane (Thermo Fisher) in degassed, nitrogen-bubbled deionized water at a concentration of 20 mg/ml (makes a 0.14 M stock solution). The solution should be used immediately. Add 70 µl of the 2-iminothiolane solution to each ml of the gelonin solution (final concentration is about 10 mM).
3. React for 1 hour at 0°C (or on ice) under a nitrogen blanket.
4. Purify the thiolated protein from unreacted Traut's reagent by gel filtration on a desalting resin using 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, containing 10 mM EDTA. The presence of EDTA in this buffer helps to prevent oxidation of the sulfhydryl groups and resultant disulfide formation. The degree of —SH modification in the purified protein may be determined using the Ellman's assay (Chapter 1, Section 4.1).
5. Concentrate the thiolated toxin to 10 mg/ml using centrifugal concentrators. Immediately use the modified protein in the conjugation reaction to prevent inactivation of 2-iminothiolane-modified molecules by recyclization (Chapter 1, Section 4.1).

Conjugation of SPDP-Activated Antibody with Thiolated Gelonin

1. Mix SPDP-activated antibody with thiolated gelonin in equal mass quantities (or equal volumes if they are at the same concentration). This ratio results in about a 5-fold molar excess of toxin over the amount of antibody.
2. React for 20 hours at 4°C under a nitrogen blanket.
3. To block unreacted sulfhydryl groups, add iodoacetamide to the solution to a final concentration of 2 mM.
4. React for an additional 1 hour at room temperature.
5. Remove unconjugated gelonin by passage of the conjugate solution over a column of immobilized protein A (Thermo Fisher). Use 2 ml of the protein A column for each 10 mg of conjugate to be purified. Equilibrate the column with 50 mM sodium phosphate, 0.15 M NaCl, pH 7.5. Apply the conjugate sample and allow it to enter the gel. Continue to wash the column with equilibration buffer while taking 2 ml fractions until baseline is reached (monitored at an absorbance of 280 nm). Unconjugated gelonin will pass through the column unretarded. Elute bound conjugate with 0.1 M acetic acid, 0.15 M NaCl. Immediately add 0.1 ml of 1 M potassium phosphate, pH 7.5 to each bound fraction for neutralization. Alternatively, gel filtration may be used to isolate the conjugate from lower-molecular-weight antibody and gelonin. A column of Sephacryl S-200 works well for this purpose.

SMPT

Succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (SMPT) is a heterobifunctional crosslinking agent similar to SPDP that contains an amine-reactive NHS ester on one end and a sulfhydryl-reactive pyridyl disulfide group on the other (Chapter 5, Section 1.2). Reaction with a sulfhydryl-containing protein results in a cleavable disulfide linkage, important for immunotoxin activity. SMPT is an analog of SPDP that differs only in its cross-bridge, which contains an aromatic ring and a hindered disulfide group (Thorpe *et al.*, 1987; Ghetie *et al.*, 1990). The spacer arm of SMPT is slightly longer than SPDP, but the presence of the benzene ring and α-methyl group adjacent to the disulfide sterically hinders the structure sufficiently to provide increased half-life of immunotoxin conjugates *in vivo*.

SMPT often is used in place of SPDP for the preparation of immunotoxin conjugates. The hindered disulfide of SMPT has distinct advantages in this regard. Thorpe *et al.* (1987) showed that SMPT conjugates had approximately twice the half-life *in vivo* as SPDP conjugates. Antibody–toxin conjugates prepared with SMPT possess a half-life *in vivo* of up to 22 hours, presumably due to the decreased susceptibility of the hindered disulfide toward reductive cleavage.

Ghetie *et al.* (1991) developed a large-scale preparation procedure for antibody–deglycosylated ricin A chain (dgA) conjugates utilizing this crosslinker. The following procedure describes a generalized method for using SMPT to prepare dgA–antibody conjugates. It is based on the Ghetie protocol, but using smaller quantities of reagents. Figure 21.8 illustrates the reactions involved in using SMPT.

Protocol

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

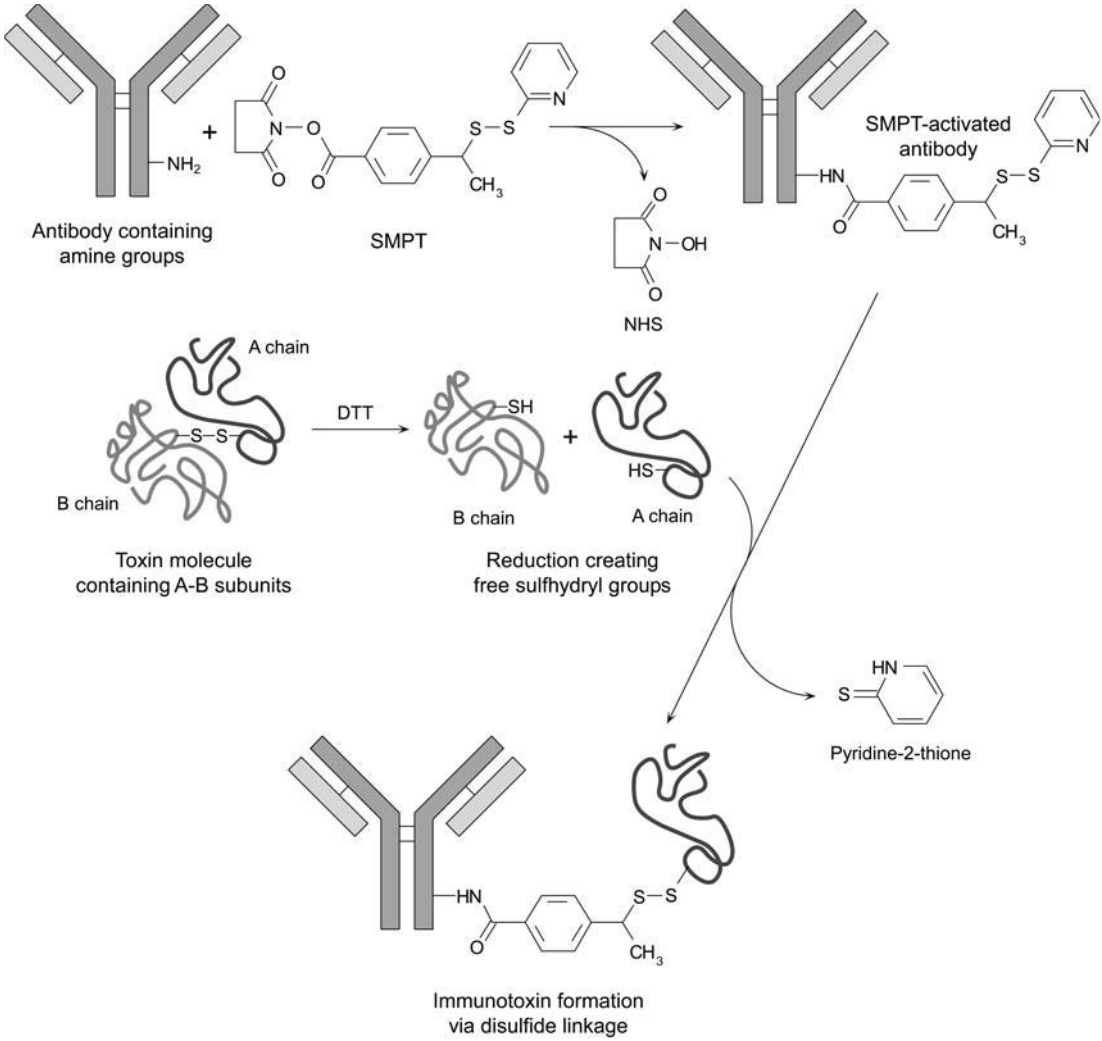

Figure 21.8 SMPT may be used to form immunotoxin conjugates by activation of the antibody component to form a thiol-reactive derivative. Reduction of an A–B toxin molecule with DTT can facilitate subsequent isolation of the A chain containing a free thiol. Mixing the A-chain containing a sulfhydryl group with the SMPT-activated antibody causes immunotoxin formation through disulfide bond linkage. The hindered disulfide of an SMPT crosslink has been found to survive *in vivo* for longer periods than conjugates formed with SPDP.

The following method calls for mixing activated antibody with ricin A chain at a ratio of 2 mg antibody per mg of A chain. Adjustments to the amount of antibody and A chain initially dissolved in the reaction buffers should be done to anticipate this ratio.

1. Dissolve the antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 10 mg/ml. If the antibody contains oligomers (as evidenced by nondenaturing electrophoresis or HPLC gel filtration analysis), then the monomeric IgG

form should be isolated by gel filtration using a column of Sephacryl S-200HR. If no oligomers are present, then omit the chromatographic purification.

2. Dissolve SMPT (Thermo Fisher) in DMF at a concentration of 4.8 mg/ml. Add 27 µl of this solution to each ml of the antibody solution. Mix gently. The final concentration of SMPT in the reaction mixture is 0.13 mg/ml, which translates into about a 4.8-fold molar excess of crosslinker over the amount of antibody present.
3. React for 1 hour at room temperature.
4. Remove unreacted SMPT and reaction by-products by gel filtration on a desalting resin. Pool fractions containing SMPT-activated antibody (the first peak eluting from the column) and concentrate them to 10 mg/ml using centrifugal concentrators with a MW cutoff of 10,000.
5. Dissolve deglycosylated ricin A chain (dgA) in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5, at a concentration of 10 mg/ml. The buffer should be degassed under vacuum and nitrogen bubbled through it to remove oxygen. Prepare half the amount of A-chain solution as the amount of antibody prepared in step 1. If the A-chain preparation is done in bulk quantities or if the protein has been stored for lengthy periods, it may be necessary to reduce the sulfhydryls with DTT prior to proceeding with the crosslinking reaction. If A-chain sulfhydryl oxidation is suspected, add 2.5 mg of DTT per ml of A-chain solution. React for 1 hour at room temperature. Purify the reduced ricin A chain by gel filtration on a desalting resin using the PBS-EDTA buffer. Apply no greater volume of sample to the gel than is represented by 5 percent of the column volume to assure good removal of excess DTT. Collect the protein and concentrate to 10 mg/ml using centrifugal concentrators.
6. Mix the reduced A-chain solution with activated antibody solution at a ratio of 2 mg of antibody per mg of A chain. Sterile filter the solution through a 0.22 µm membrane, and react at room temperature under nitrogen for 18 hours.
7. To block excess pyridyl disulfide active sites on the antibody, add cysteine to a final concentration of 25 µg/ml. React for an additional 6 hours at room temperature.
8. To isolate the conjugate, apply the immunotoxin solution to a column of Sephacryl S-200HR. Collect the peaks with MW between 150,000 and 210,000. Further purification to remove excess unconjugated antibody can be done on a column of immobilized Cibacron Blue (available commercially (Thermo Fisher) or for column preparation, see Hermanson *et al*., 1992). Equilibration of the column with 50 mM sodium borate, 1 mM EDTA, pH 9.0, will bind the conjugate, but not free antibody. Elution of purified immunotoxin conjugate can be done with 50 mM sodium borate, 1 mM EDTA, 0.5 M NaCl, pH 9.0 (see Ghetie *et al*., 1991).

3-(2-Pyridyldithio) Propionate

A lesser-used reagent to introduce sulfhydryl-reactive pyridyl disulfide groups is 3-(2-pyridyldithio)propionate (PDTP), the acid precursor of SPDP containing no NHS ester group on the carboxylate. Sulfhydryl interchange reaction at the pyridyl dithiol end results in the formation of a disulfide linkage with —SH-containing molecules. The carboxylate end is not further derivatized to contain a reactive species, but may be coupled to amines by the carbodiimide reaction (Chapter 3, Section 1). Reaction of PDTP with an antibody molecule in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) results in the formation of amide linkages with the active pyridyl disulfide groups still available for coupling to sulfhydryl-containing

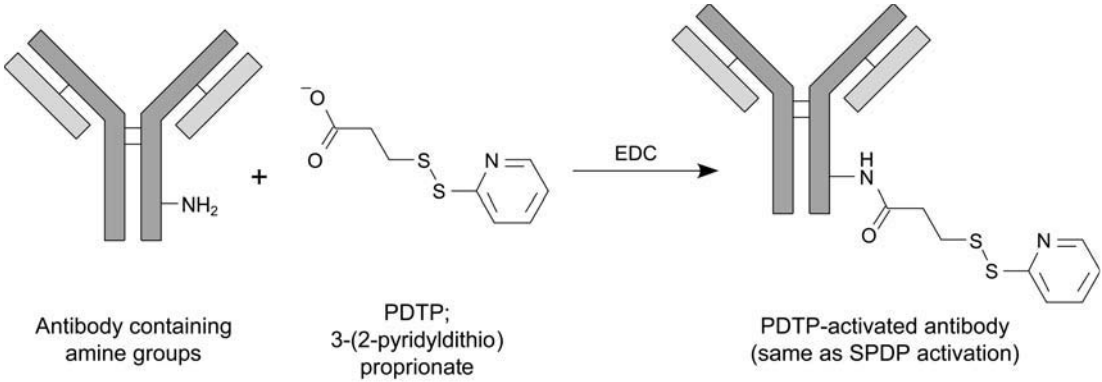

Figure 21.9 PDTP may be used to modify antibody molecules using a carbodiimide reaction with EDC. The derivative is the same as that obtained using SPDP activation and is highly reactive toward sulfhydryls.

toxins (Figure 21.9). Mixing the PDTP–antibody with purified ricin A chain results in disulfide crosslinks identical to those obtained using SPDP as the crosslinker (Jansen *et al*., 1980; Gros *et al*., 1985). PDTP also has been used to activate transferrin to contain reactive pyridyl dithiol groups for conjugation to ricin A-chain molecules (Raso and Basala, 1984, 1985).

Since activated molecules and crosslinks formed between two species are identical to those formed using SPDP, it is of little advantage to use PDTP. Furthermore, an EDC-mediated reaction of the carboxylate end of the crosslinker with amine groups on proteins can cause concomitant zero-length crosslinking and polymerization of protein molecules. For these reasons, SPDP is the better choice for preparing conjugates.

Use of Cystamine, Ellman's Reagent, or S-Sulfonates

Other reagent systems can be used to form disulfide linkages between antibody and toxin molecules in immunotoxin conjugates. Cystamine can be incorporated into proteins by reaction of its terminal amines with the carboxylates on the proteins via the carbodiimide reaction (Chapter 3, Section 1). The resultant modifications contain disulfide linkages that can undergo disulfide interchange reactions with other sulfhydryl-containing molecules (Chapter 1, Section 4.1). For instance, a cystamine-modified-targeting component, such as an antibody, can be mixed with the reduced A chain of a toxin molecule to cause conjugate formation through the creation of a disulfide bond (Figure 21.10) (Oeltmann and Forbes, 1981). Epidermal growth factor was modified with cystamine and coupled with reduced diphtheria toxin using this approach (Shimisu *et al*., 1980).

Similarly, Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] can be used to activate one thiol-containing molecule by disulfide exchange and subsequently used to couple to a second sulfhydryl-containing molecule by the same mechanism (Chapter 1, Section 5.2) (Figure 21.11). The disulfide of Ellman's reagent readily undergoes disulfide exchange with a free sulfhydryl to form a mixed disulfide with simultaneous release of one molecule of the highly chromogenic 5-sulfido-2-nitrobenzoate, also called 5-thio-2-nitrobenzoic acid (TNB). The intense yellow color produced by the TNB anion can be measured by its absorbance at 412 nm. Thus, the efficiency of conjugation can be determined spectrophotometrically using this procedure (Pirker *et al*.,

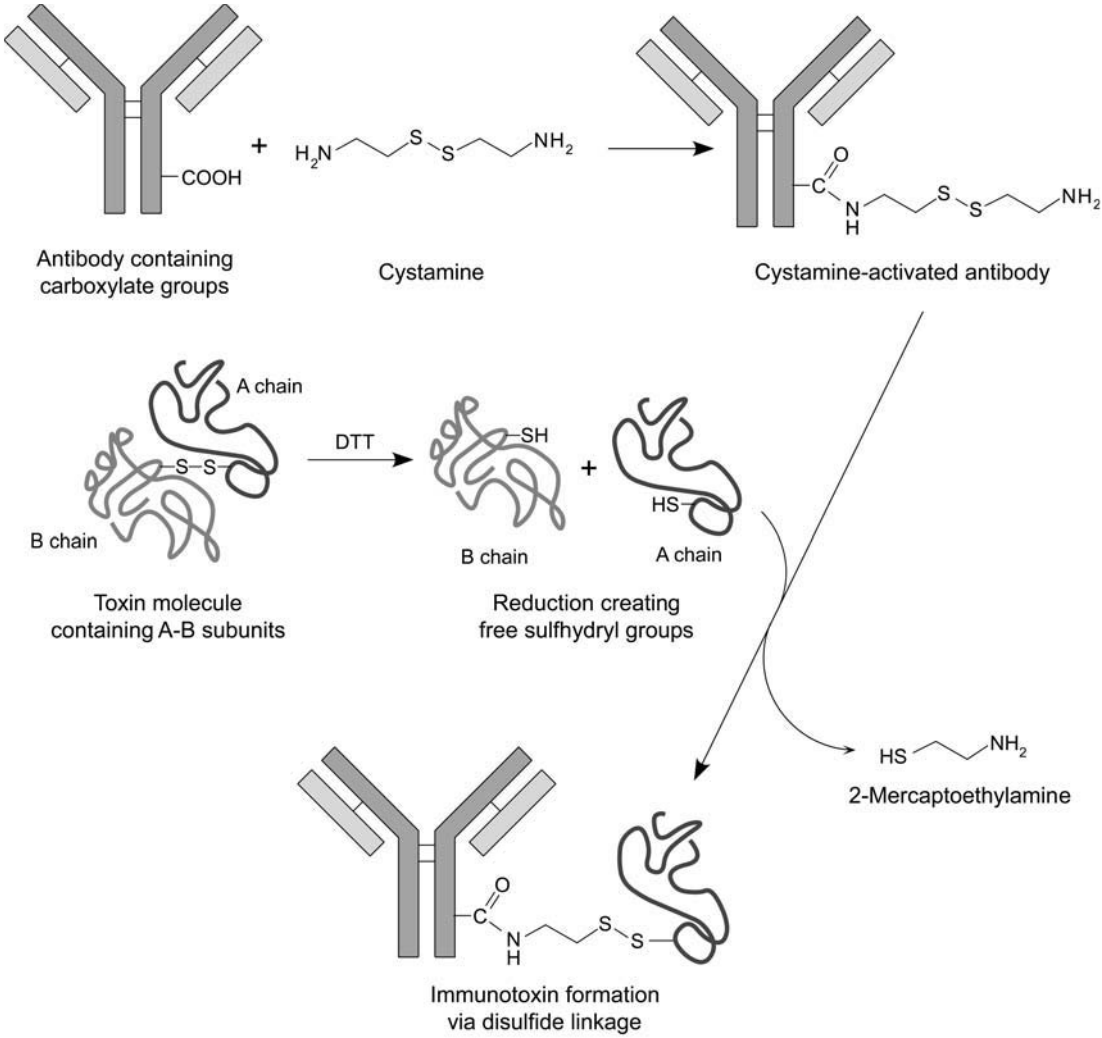

Figure 21.10 Cystamine may be used to make immunotoxin conjugates by a disulfide interchange reaction. Modification of antibody molecules using an EDC-mediated reaction creates a sulfhydryl-reactive derivative. A-chain toxin subunits containing a free thiol can be coupled to the cystamine-modified antibody to form disulfide crosslinks.

1986; Fitzgerald *et al.*, 1988). A method for the large-scale conjugation of Fab′ fragments containing an available sulfhydryl group and deglycosylated ricin A chain (also containing an —SH group) were developed using Ellman's reagent as the crosslinker (Ghetie *et al.*, 1988).

A final method of forming disulfide crosslinks between toxins and targeting molecules is the use of S-sulfonate formation using sodium sulfite (Na_2SO_3) in the presence of sodium tetrathionate ($Na_2S_4O_6$). Tetrathionate reacts with sulfhydryls to form sulfenylthiosulfate intermediates (section 1.1.5.2). These derivatives are reactive toward other thiols to create disulfide linkages

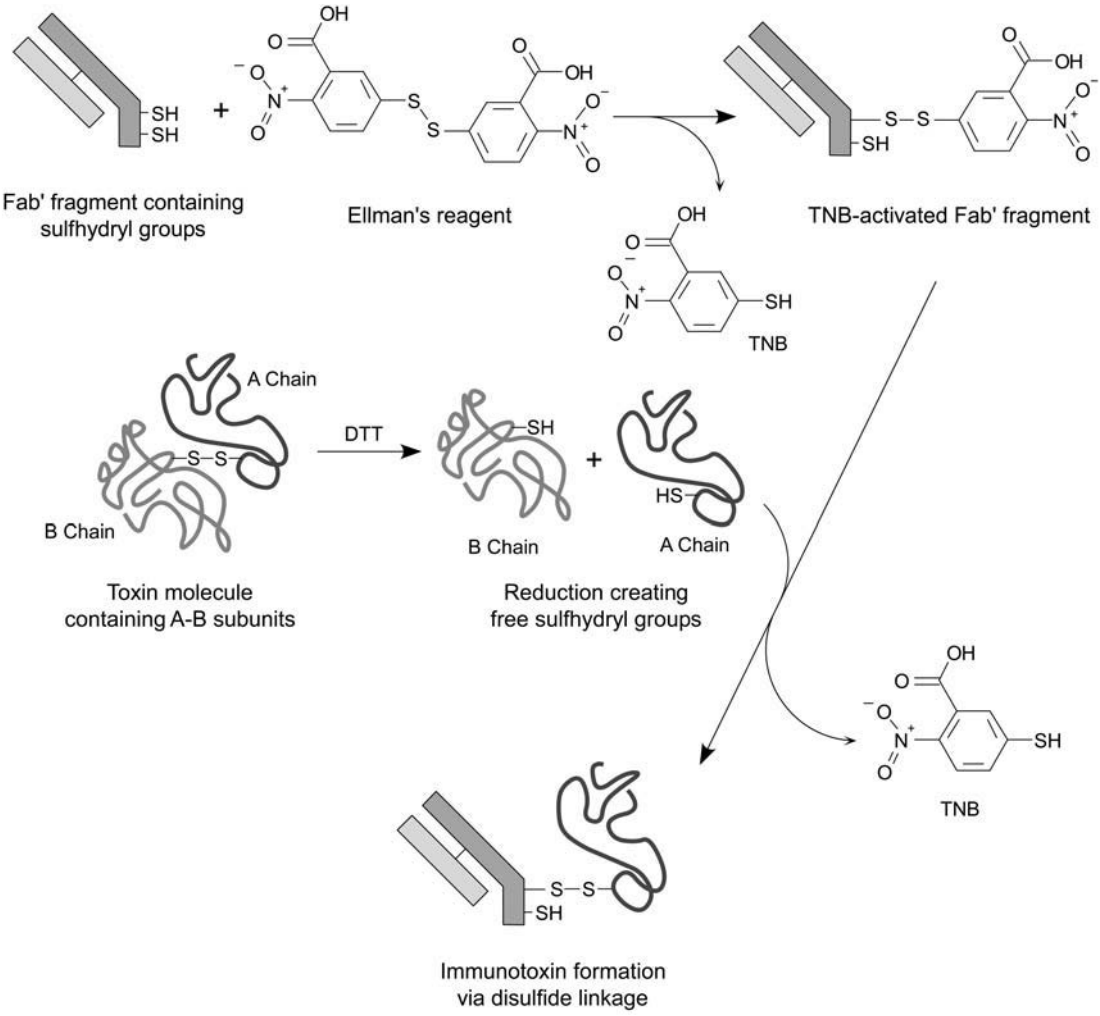

Figure 21.11 Fab′ antibody fragments containing free thiols can be activated with Ellman's reagent to form a sulfhydryl-reactive derivative. A-chain toxin subunits containing a free thiol group may be coupled to the activated Fab′ molecule to produce an immunotoxin complex.

rapidly. Sulfite ions react with disulfides to form S-substituted thiosulfates, also known as S-sulfonates, and a thiol. The combination of these reagents results in the transformation of available thiols and disulfides into reactive S-sulfonates that can be used to crosslink with sulfhydryl-containing molecules. S-sulfonate conjugation can be used to conjugate the A chain of toxin molecules with sulfhydryl-containing Fab′ fragments with good efficiency (Masuho *et al.*, 1979). Although the use of these alternative disulfide generating agents has proven successful in some applications, pyridyl disulfide-containing crosslinkers, as discussed previously, are more common for producing immunotoxin conjugates.

2.2. Preparation of Immunotoxin Conjugates via Amine- and Sulfhydryl-Reactive Heterobifunctional Crosslinkers

Other forms of heterobifunctional crosslinkers that can be used for this purpose are the amine- and sulfhydryl-reactive agents that produce a thioether bond with —SH-containing molecules (Chapter 5, Section 1). The amine-reactive end of these crosslinkers is usually an NHS ester group that can form a stable amide bond with amine-containing proteins. One of two main reactive groups usually are used on the sulfhydryl-reactive end: an iodoacetyl group which couples to sulfhydryls with loss of HI or a maleimide group which undergoes a double bond addition reaction with —SH groups (see Chapter 2, Sections 2.1 and 2.2).

Since this type of crosslinker forms non-cleavable thioether bonds between toxin molecules and the targeting component of the conjugates, they are not appropriate for use with A-chain or single-chain toxins. This is because the crosslinker will not allow the conjugated A chain to break free of the antibody by disulfide reduction after docking at the cellular target. Since release of the A chain is a prerequisite to ribosomal inactivation, such conjugates will prove to be ineffective cytotoxic agents. One report found a 1,000-fold increase in cytotoxicity when an immunotoxin-containing PAP or gelonin was prepared using a cleavable disulfide linker as opposed to a non-cleavable thioether linkage (Lambert *et al*., 1985).

To make effective immunotoxin conjugates using the following crosslinkers, it is necessary to crosslink intact A–B toxins to antibodies, not single chain or A-chain toxins. Using intact two-subunit toxins allows the A chain to break free of the complex and perform its cytotoxic duties upon entering the target cell. Two main criteria are especially important when using A–B toxin conjugates: the B-chain binding site must be blocked or inoperative in the final immunotoxin complex to prevent nonspecific cell death, and secondly, the two subunits of the toxin must not be covalently crosslinked by the conjugation procedure, precluding them from being separated *in vivo*.

Fortunately, satisfying these criteria is not difficult. The heterobifunctional crosslinkers described in this section are sufficiently controllable as to prevent A–B chain crosslinking. In addition, during the conjugation process, the B-chain binding site often becomes inactivated or physically blocked by the attached antibody molecule. Subsequent cleanup of the conjugate using affinity chromatography over a column containing an immobilized sugar can completely eliminate any potential nonspecificity contributed by the B chain in the final preparation.

It should be noted that the use of the following crosslinkers to create other forms of toxic conjugates for cancer therapy is not restricted by the disulfide bridge requirement (Trail *et al*., 1993; Willner *et al*., 1993). Drug–toxin conjugates, hormonotoxins, lymphokine– or growth factor–toxin conjugates all can be made using nonreversible thioether linkages without difficulty.

SIAB

N-Succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) is a heterobifunctional crosslinker containing amine-reactive and sulfhydryl-reactive ends (Chapter 5, Section 1.5). The NHS ester on one end of the reagent can be used to couple with primary amine-containing molecules, forming stable amide linkages (Chapter 2, Section 1.4). The other end contains an iodoacetyl group that is specific for coupling to sulfhydryl residues, potentially creating stable thioether bonds (Chapter 2, Section 2.1).

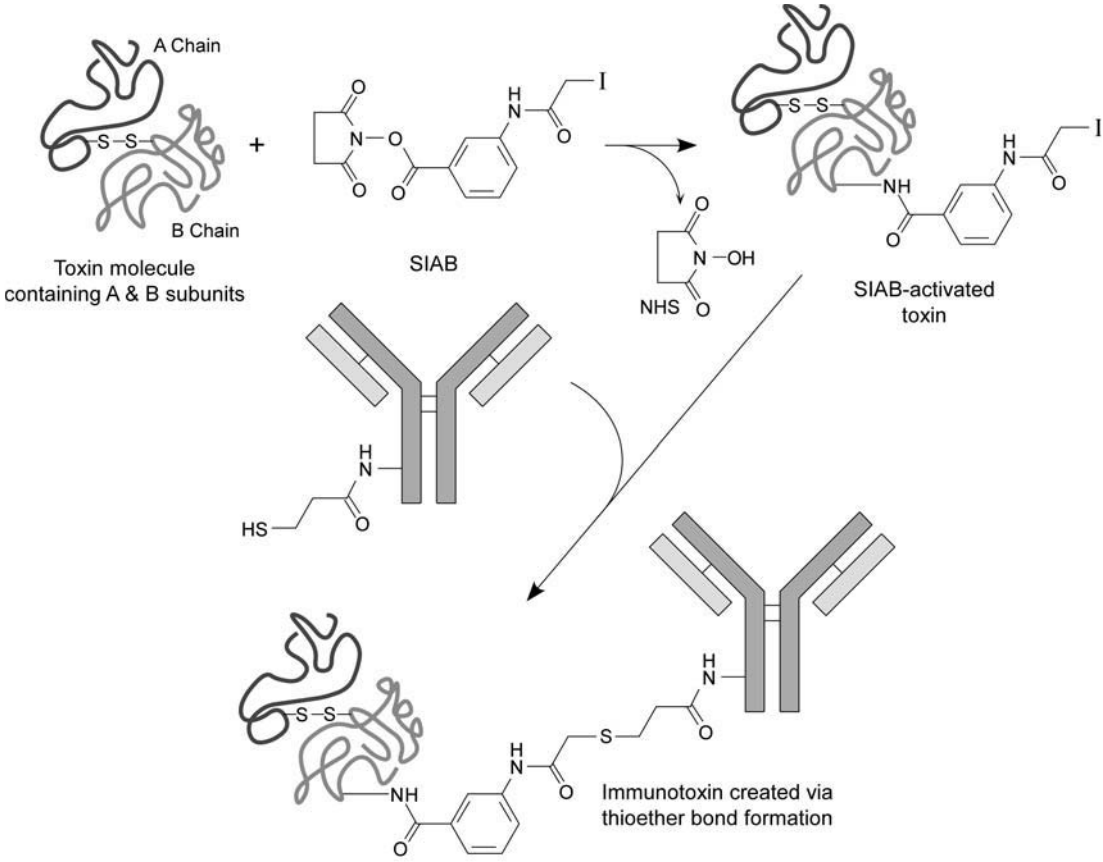

Figure 21.12 SIAB can be used to activate toxin molecules for coupling with sulfhydryl-containing antibodies. In this case, the antibody molecule is thiolated using SATA and deprotected to reveal the free sulfhydryl. Reaction with the SIAB-activated toxin forms the final conjugate by thioether bond formation.

Conjugations using SIAB to create immunotoxins can be done by first reacting the NHS ester end of the crosslinker with available amine groups on the antibody and then coupling to a thiolated toxin dimer—or by first reacting it with the toxin and coupling to a thiolated antibody (Figure 21.12). Thiolation of the secondary component is usually done with SPDP or 2-iminothiolane. Other crosslinkers containing an iodoacetyl group can be used in a similar fashion.

Conjugations with iodoacetyl crosslinkers have been done using ricin and CVF (Thorpe *et al*., 1984; Vogel, 1987; Myers *et al*., 1989). The following generalized protocol for using SIAB is based on the method of Cumber *et al*. (1985).

Protocol

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

To prepare an antibody–ricin conjugate using this protocol, 2.25 mg of antibody is needed for every mg of toxin.

Activation of Toxin with SIAB

1. Dissolve intact ricin in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration of 10 mg/ml.
2. Dissolve SIAB (Thermo Fisher) in DMSO at a concentration of 1.4 mg/ml. Prepare fresh and protect from light to avoid breakdown of the active halogen group.
3. Add 160 µl (225 µg) of the SIAB solution to each ml of the ricin solution.
4. React for 30 minutes at room temperature in the dark.
5. Remove excess crosslinker from the activated ricin by gel filtration using a desalting resin.
6. Concentrate the purified, SIAB-activated toxin to 10 mg/ml using centrifugal concentrators with a MW cutoff of 10,000. Protect the activated toxin from light to prevent degradation of the iodoacetyl-reactive group.

Thiolation of Specific Antibody Molecule with SPDP

1. Dissolve the antibody to be conjugated in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, at a concentration 10 mg/ml. Use 2.25 mg of antibody per mg of toxin to be conjugated.
2. Dissolve SPDP (Thermo Fisher) in DMF at a concentration of 3 mg/ml. Add 24 µl of this solution to each ml of the antibody solution with gentle mixing to effect complete dissolution.
3. React for 30 minutes at room temperature.
4. Remove excess crosslinker by gel filtration using a column of desalting resin. Perform the chromatography using 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5. The buffer should be degassed under vacuum and nitrogen bubbled through it to remove oxygen. The presence of EDTA stabilizes the free sulfhydryls formed in the following steps against metal-catalyzed oxidation.
5. Concentrate the fractions containing protein from the gel filtration step to 10 mg/ml using a centrifugal concentrator (MW cutoff of 10,000).
6. To reduce the pyridyl dithiol groups and create reactive sulfhydryls, dissolve DTT (Thermo Fisher) in water at a concentration of 17.2 mg/ml and immediately add 500 µl of this solution to each ml of concentrated antibody solution. Mix to dissolve and react for 30 minutes at room temperature.
7. Remove excess DTT by gel filtration using the same buffer as in step 4. Pool the fractions containing protein and concentrate to 10 mg/ml.

Conjugation of SIAB-Activated Toxin with Thiolated Antibody

1. Mix activated toxin from part A with thiolated antibody from part B at a ratio of 2.25 mg of antibody per mg of toxin. Protect the solution from light.
2. React for 18 hours at room temperature in the dark.
3. To block unreacted sulfhydryl groups, add iodoacetamide to the solution to a final concentration of 2 mM. React for an additional 1 hour at room temperature.
4. Isolation of the ideal 1:1 or 1:2 antibody–toxin conjugate can be done by gel filtration separation using a column of Sephacryl S-300.

SMCC

Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is a crosslinker with significant utility in protein conjugation (Chapter 5, Section 1.3). It is a popular choice among heterobifunctional reagents, especially for the preparation of antibody–enzyme and hapten–carrier conjugates (Hashida and Ishikawa, 1985; Dewey *et al*., 1987). The NHS ester end of the reagent can react with primary amine groups on proteins to form stable amide bonds. The maleimide end of SMCC is specific for coupling to sulfhydryls when the reaction pH is in the range of 6.5–7.5 (Smyth *et al*., 1964). The nature of the reactive groups of SMCC allow for highly controlled crosslinking procedures to be performed wherein the resulting products can be closely limited to a 1:1 ratio in the final complex. Thus, low-molecular-weight conjugates can be made which make ideal reagents for *in vivo* purposes.

However, since SMCC forms nonreversible thioether linkages with sulfhydryl groups, it only can be used in the preparation of immunotoxins if intact A–B toxins are employed in the conjugate. In such conjugates, the A chain still have the potential for reductive release from the B-chain subunit after cellular docking and internalization. Immunotoxins prepared with A-chain or single-subunit toxins will not display cytotoxicity if crosslinked with SMCC, since the crosslinker does not create cleavable disulfide bonds upon conjugation.

SMCC has been used to prepare immunotoxins with CVF (Vogel, 1987) and was compared to other crosslinkers in the preparation of gelonin and PAP conjugates (Lambert *et al*., 1985).

The following protocol is a suggested method for the conjugation of SMCC-activated antibodies with 2-iminothiolane-modified, intact toxin molecules (Figure 21.13). It utilizes the water-soluble analog of SMCC, sulfo-SMCC, which contains a negatively charged sulfonate group on its NHS ring.

Protocol

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

Note: This protocol requires mixing activated antibody with thiolated toxin at a ratio of 2.25 mg of antibody per mg of toxin. This ratio should be taken into account before starting the reactions.

Activation of Antibody with Sulfo-SMCC

1. Dissolve 10 mg of specific antibody in 1 ml of 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. Weigh out 2 mg of sulfo-SMCC (Thermo Fisher) and add it to the above solution. Mix gently to dissolve. To aid in measuring the exact quantity of crosslinker, a concentrated stock solution may be made in water and an aliquot equal to 2 mg transferred to the reaction solution. If a stock solution is made, it should be dissolved rapidly and used immediately to prevent extensive hydrolysis of the active ester. Alternatively, a stock solution of sulfo-SMCC may be prepared in DMSO and an aliquot added to the aqueous reaction.
3. React for 1 hour at room temperature.
4. Immediately purify the maleimide-activated protein by applying the reaction mixture to a desalting column. Do not dialyze the solution, since the maleimide activity will be lost over

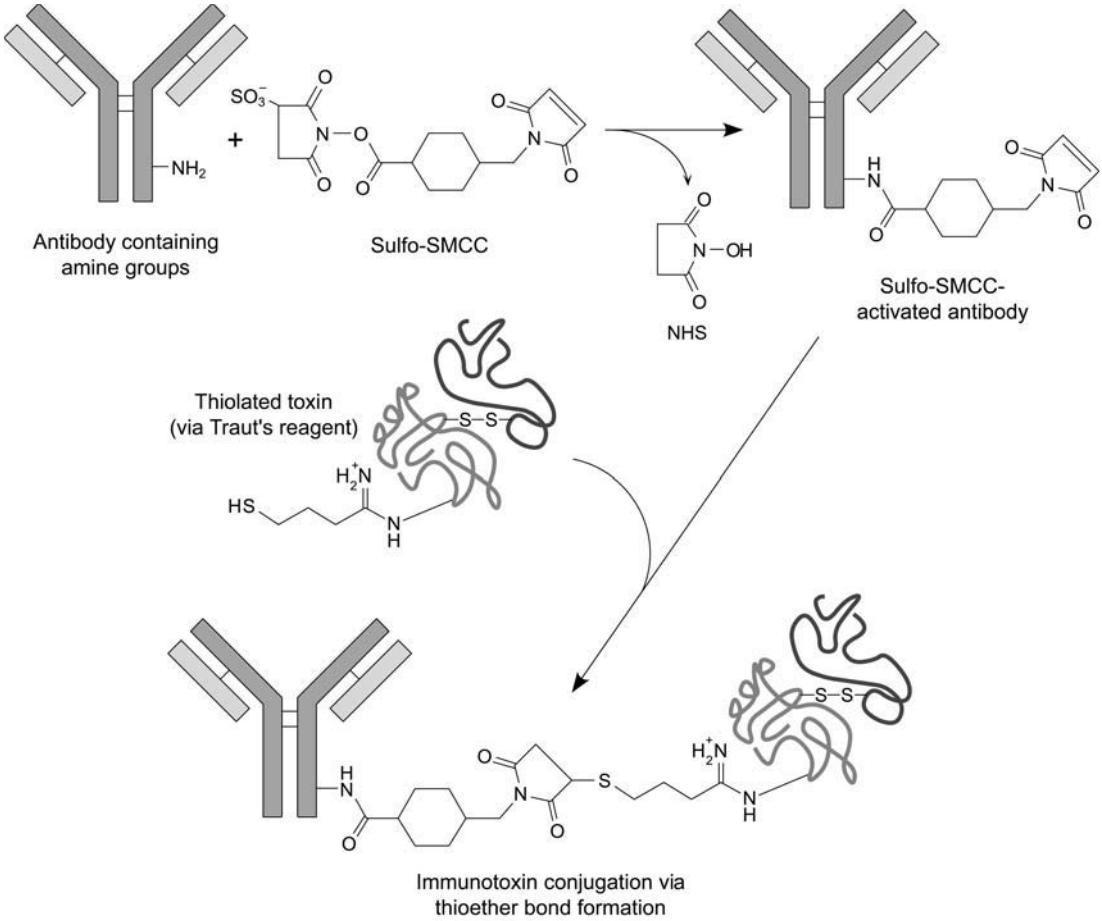

Figure 21.13 Sulfo-SMCC may be used to activate antibody molecules for coupling to thiolated toxin components. An intact A–B toxin molecule can be modified to contain sulfhydryls by treatment with 2-iminothiolane. Thiolation with this reagent retains the cytotoxic properties of the toxin while generating a sulfhydryl for conjugation. Reaction of the thiolated toxin with the maleimide-activated antibody creates the immunotoxin through thioether bond formation.

the time course required to complete the operation. To obtain good separation between the protein peak (eluting first) and the peak representing excess reagent and reaction by-products (eluting second), the applied sample size should be no more than 5–8 percent of the column bed volume.

5. Collect the peak containing the activated antibody (eluting first) and concentrate to 10 mg/ml using centrifugal concentrators. Use immediately for conjugating to a thiolated toxin.

Thiolation of Intact A–B toxin

1. Dissolve the toxin (e.g., intact ricin) at a concentration of 10 mg/ml in 50 mM triethanolamine hydrochloride, pH 8.0, containing 10 mM EDTA. The buffer should be

degassed under vacuum and bubbled with nitrogen to remove oxygen that may cause sulfhydryl oxidation after thiolation.
2. Dissolve 2-iminothiolane in degassed, nitrogen-bubbled deionized water at a concentration of 20 mg/ml (makes a 0.14 M stock solution). The solution should be used immediately. Add 70 µl of the 2-iminothiolane solution to each ml of the toxin solution (final concentration is about 10 mM).
3. React for 1 hour at 0°C (on ice) under a nitrogen blanket.
4. Purify the thiolated toxin from unreacted Traut's reagent by gel filtration using 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, containing 10 mM EDTA. The presence of EDTA in this buffer helps to prevent oxidation of the sulfhydryl groups with resultant disulfide formation. The degree of —SH modification in the purified protein may be determined using the Ellman's assay (Chapter 1, Section 4.1).
5. Concentrate the thiolated toxin to 10 mg/ml using centrifugal concentrators.

Conjugation of SMCC-Activated Antibody with Thiolated Toxin

1. Mix the thiolated toxin with SMCC-activated antibody at a ratio of 2.25 mg of antibody per mg of toxin. Protect the solution from light.
2. React for 18 hours at room temperature.
3. To block unreacted sulfhydryl groups, add iodoacetamide to the solution to a final concentration of 2 mM. React for an additional 1 hour at room temperature.
4. Isolation of the ideal 1:1 antibody–toxin conjugate can be done by gel filtration separation using a column of Sephacryl S-300.
5. Removal of nonspecific binding potential in the B chain must be done before using an A–B intact toxin conjugate *in vivo*. A large proportion of the binding sites on the B chains usually are blocked during the above conjugation process, and the galactose binding potential is significantly impaired. Further purification to remove conjugates that have galactose binding potential can be done on an acid-treated agarose chromatography column (which contains galactose residues) or on a column of asialofetuin bound to agarose (Cumber *et al.*, 1985). Conjugate fractions that do not bind to both affinity gels contain no nonspecific binding potential toward non-targeted cells.

MBS

m-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) is a heterobifunctional crosslinking agent containing an NHS ester and a maleimide group. The NHS ester can react with primary amines in proteins and other molecules to form stable amide bonds, while the maleimide end reacts with sulfhydryl groups to create stable thioether linkages (Chapter 5, Section 1.4). The reagent can be used in many different conjugation protocols to crosslink amine-containing proteins with sulfhydryl-containing proteins. Since the thioether bond formed at the maleimide end is nonreversible, MBS can be used for immunotoxin preparation only if the conjugate involves crosslinking intact A–B toxins with antibody molecules. Using intact toxins (as opposed to single-chain or A-chain isolates), the A chain still is able to release from the complex after cellular docking and inactivate ribosomal activity (Youle and Nevelle, 1980; Dell'Arciprete *et al.*, 1988; Myers *et al.*, 1989).

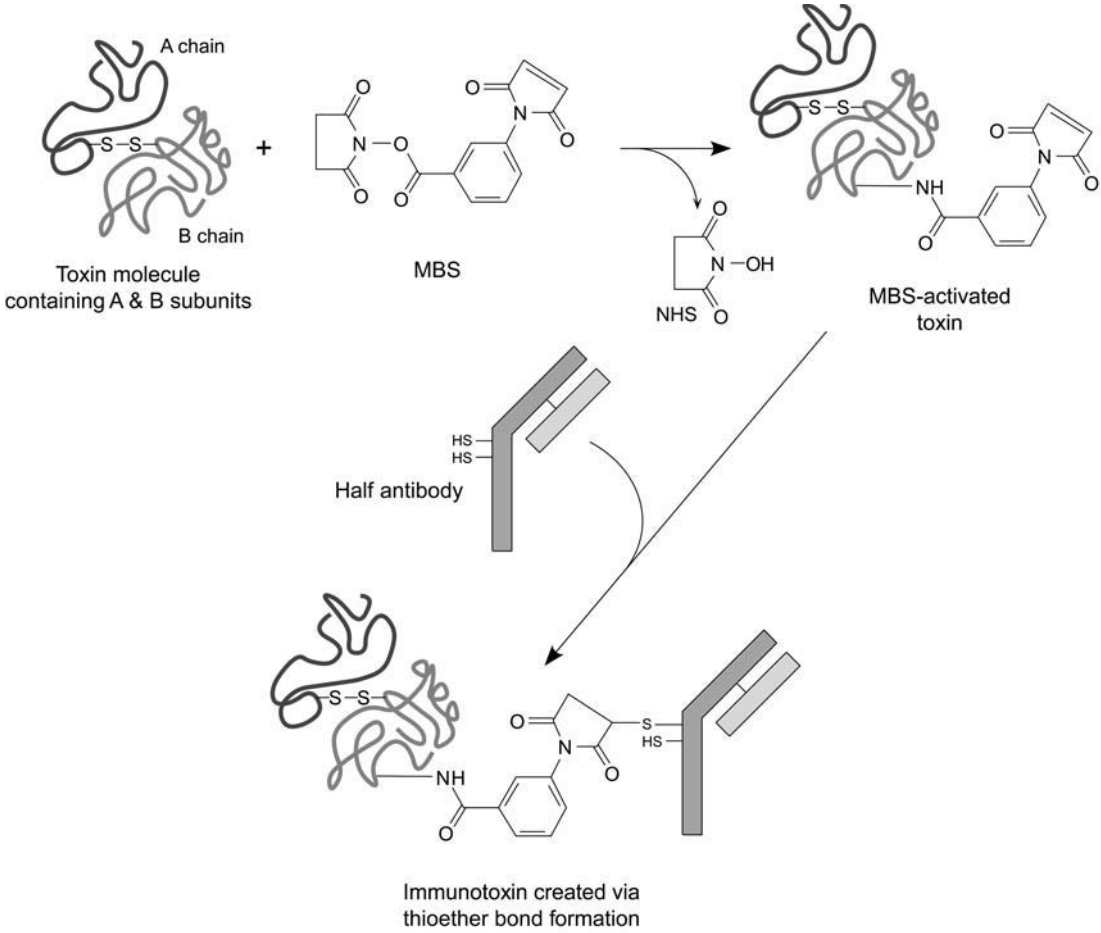

Figure 21.14 Activation of an intact A–B toxin molecule with MBS with subsequent conjugation with a reduced antibody fragment to produce an immunotoxin.

MBS contains a benzoic acid derivative as its cross-bridge. In many applications involving NHS–maleimide crosslinkers, non-aromatic cross-bridges are considered superior to aromatic ones. This is reflected in the stability of the maleimide group to hydrolysis prior to conjugating with a sulfhydryl group. For immunotoxin preparation, however, aromatic maleimides resulted in better conjugate yield and more potent cytotoxic effects when compared to aliphatic ones (Myers *et al.*, 1989). MBS, therefore, may be a crosslinker of choice when making conjugates with intact toxin molecules.

The following protocol is adapted from Myers *et al.* (1989). It involves activation of ricin with MBS and conjugation with a partially reduced antibody (Figure 21.14).

Protocol

Caution: toxin molecules are dangerously toxic even in small amounts. Use extreme care in handling.

This method uses a molar ratio of 15:1 for ricin:antibody. This requires 6.24 mg of ricin per mg of antibody. This ratio should be considered when determining how much starting materials to use for each step.

Activation of Ricin with MBS

1. Dissolve ricin at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5.
2. Dissolve MBS (Thermo Fisher) in DMF at a concentration of 2 mg/ml.
3. Add 76 µl of the MBS solution to each ml of the ricin solution. This represents a 3:1 molar ratio of crosslinker to protein.
4. React for 30 minutes at room temperature.
5. Immediately purify the MBS-activated toxin by gel filtration using a column of desalting resin. Apply no more sample than represents 5–8 percent of the gel volume. Isolate the protein peak by its absorbance at 280 nm and concentrate to 10 mg/ml using centrifugal concentrators with a MW cutoff of 10,000.

Partial Reduction of Antibody with DTT

1. Dissolve the antibody in 0.1 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.5, at a concentration of 10 mg/ml.
2. Add DTT to a final concentration of 50 mM.
3. Reduce for 30 minutes at room temperature.
4. Purify the reduced antibody using gel filtration on a column of Sephadex G-25. Concentrate the protein to 10 mg/ml using centrifugal concentrators.

Conjugation of MBS-Activated Ricin with Partially Reduced Antibody

1. Mix the MBS-activated ricin with the partially reduced antibody in a molar ratio of 15:1 (or 6.24 mg activated ricin per mg of reduced antibody). This represents a volume ratio (at 10 mg/ml for both proteins) of 1 ml ricin solution mixed with 160 µl antibody solution.
2. React for 18 hours at room temperature.
3. Purification of the immunotoxin conjugate from unconjugated ricin can be done using a column of TSK3000 SW (Toya Soda, Japan) according to the method of Myers *et al.* (1989).
4. Removal of nonspecific binding potential in the B chain must be done before using an A–B intact toxin conjugate *in vivo*. See step 5 of the MBS conjugation protocol discussed previous to this section.

SMPB

Succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB), is a heterobifunctional analog of MBS containing an extended cross-bridge (Chapter 5, Section 1.6). The crosslinker has an amine-reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on the other. Conjugates formed using SMPB thus are linked by stable amide and thioether bonds.

As in the case of MBS, discussed previously, SMPB was found to be more effective than aliphatic crosslinkers in producing immunotoxin conjugates with ricin that have high yields of cytotoxicity (Myers *et al*., 1989). This was attributed to the reagent's aromatic ring structure. A comparison with SPDP produced immunotoxin conjugates concluded that SMPB formed more stable complexes that survive in serum for longer periods (Martin and Papahadjopoulos, 1982).

The method for the preparation of immunotoxins with SMPB is identical to that used for MBS (above). Since the thioether bonds formed with sulfhydryl-containing molecules are non-cleavable, A-chain isolates or single-chain toxin molecules can not be conjugated with antibodies with retention of cytotoxicity. Only intact A–B toxin molecules may be used with this crosslinker, since the A chain still is capable of being reductively released from the complex.

2.3. Preparation of Immunotoxin Conjugates via Reductive Amination

Conjugations involving aldehyde groups and amine-containing molecules can be done through Schiff base formation with subsequent reduction using sodium cyanoborohydride to form stable secondary amine linkages (Chapter 2, Section 5.3). Carbohydrates, glycoproteins, and other polysaccharide-containing molecules can be oxidized to contain aldehyde residues by sodium periodate or specific oxidases (Chapter 1, Section 4.4). Some antibodies and toxin molecules are glycoproteins and contain sufficient carbohydrate to be utilized for reductive amination crosslinking.

A second method of immunotoxin preparation by reductive amination involves the use a polysaccharide spacer. Soluble dextran may be oxidized with periodate to form a multifunctional crosslinking polymer. Reaction with antibodies and cytotoxic molecules in the presence of a reducing agent forms multivalent immunotoxin conjugates. The following sections discuss these options.

Periodate Oxidation of Glycoproteins Followed by Reductive Conjugation

Antibody molecules usually contain carbohydrate in their Fc regions. Similarly, many toxins, such as ricin and abrin, are glycoproteins that contain abundant polysaccharide. These carbohydrate residues can be oxidized with 10 mM sodium periodate to form reactive aldehyde groups capable of being conjugated with primary amines (Chapter 1, Section 4.4). Mixing an aldehyde-containing glycoprotein with another amine-containing molecule in the presence of sodium borohydride or sodium cyanoborohydride reduces the intermediate Schiff bases that are formed to stable secondary amine bonds. Since functional groups on the antibody and the toxin components are the only ones necessary for this type of conjugation strategy, it is often referred to as a zero-length crosslinking procedure (Chapter 3). In other words, no additional crosslinking reagents are introduced into the site of the crosslink. This method of conjugation is used with great success in the formation of antibody–enzyme conjugates, especially using the glycosylated enzyme, horseradish peroxidase (HRP) (Chapter 26, Section 1.1).

The disadvantage of this type of conjugation approach for producing immunotoxins is that many of the monoclonal antibodies or antibody fragments used for immunotoxin conjugation are devoid of carbohydrate. Especially when using small Fv fragments or single-chain antibodies produced by recombinant techniques, there are typically no polysaccharide portions attached to them. In this case, creation of aldehydes on the targeting component is not possible. In addition, not all toxin molecules contain carbohydrate. Ricin, abrin, and CVF are glycoproteins and can be oxidized and coupled to antibodies without difficulty (Olsnes and Pihl,

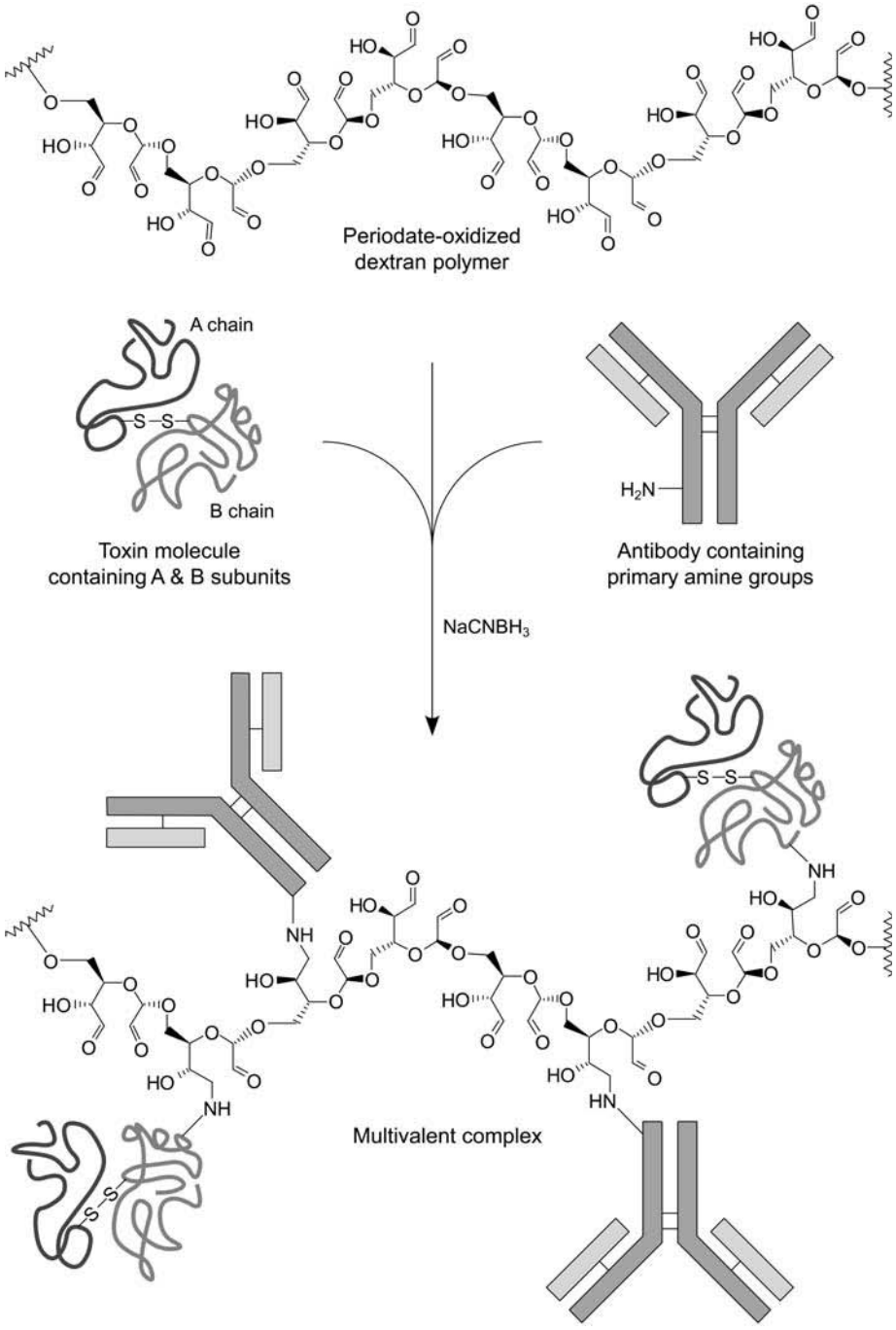

Figure 21.15 A periodate-oxidized dextran polymer may be reacted with both an antibody and an intact toxin component using reductive amination to form a multivalent immunotoxin complex.

1982a, b; Vogel and Muller-Eberhard, 1984). However, it is not well known if immunotoxin conjugates formed by this procedure retain their ability to inhibit ribosomal activity.

Suggested procedures for using reductive amination techniques may be found in Chapter 1, Section 4.4 and Chapter 3, Section 4.

Periodate-Oxidized Dextran as Crosslinking Agent

Dextran polymers consist of glucose residues bound together predominantly in α-1,6 linkages. The main repeating unit is an isomaltose group. Most preparations of dextran contain some branching, mainly incorporating 1,2, 1,3, and 1,4 linkages. The degree of branching is characteristic of its source—the strain and species of yeast or bacteria from which the dextran originated. The terminating monosaccharide in a dextran polymer is often a fructose group. Dextrans of MW 10,000–40,000 provide long, hydrophilic arms that can accommodate multiple attachment points for macromolecules along their length. Soluble dextrans can be oxidized in aqueous solution to create numerous aldehyde residues suitable for use in reductive amination techniques (Hurwitz *et al*., 1978, 1985; Manabe *et al*., 1983; Sela and Hurwitz, 1987). Periodate oxidation results in the cleavage of the carbon–carbon bonds between the No. 2 and 3 carbons within each monosaccharide unit of the chain, transforming the associated hydroxyl groups into aldehydes (Chapter 1, Section 4.4).

Periodate-oxidized dextran can be used as a protein modification or crosslinking agent (Chapter 25, Section 2). Conjugation of antibody molecules to toxins can be done with dextran to produce immunotoxins suitable for *in vivo* administration. Mixing of the antibody and toxin together with the oxidized dextran under alkaline conditions results in the formation of Schiff base interactions with the amines on both proteins. Reduction of these Schiff base linkages with sodium borohydride or sodium cyanoborohydride results in stable amide bonds, covalently attaching multiple antibody and toxin molecules along the length of the polysaccharide chain (Figure 21.15).

Chemoimmunoconjugates consisting of drugs attached to antibody-targeting molecules also can be formed using oxidized dextran carriers. Cancer therapeutic agents such as adriamycin, bleomycin, and daunomycin can be coupled to the oxidized dextran through their amine groups. After formation of Schiff base linkages between these drugs and the carrier, the antibody is added and a reducing agent used to create the final amide bond linkages (Sela and Hurwitz, 1987). The dextran backbone provides many more drug molecules associated with each antibody than could be accomplished by direct conjugation to the antibody itself.

Although dextran can be a versatile crosslinking agent for the preparation of many forms of macromolecular conjugates, immunotoxin conjugation may be impeded by the nonreversibility of the multiple amide bond linkages formed during reductive amination. Certainly, only intact A–B toxins have a chance of succeeding with this method, since A-chain or single-subunit toxins would not be capable of release from the complex after cellular docking. Even intact two-subunit toxins, however, may not be capable of releasing an A-chain unit, due to the multivalent nature of the oxidized dextran linker. For this reason, activated dextran may be more useful for constructing antibody conjugates consisting of some cytotoxic component other than protein toxins—for example, drug, hormone, or radioactive complexes.

Methods for using oxidized dextran, including reductive amination techniques, can be found in Chapter 1, Section 4.4, Chapter 3, Section 4, and especially Chapter 25, Section 2). Reference also should be made to the use of dendrimers as carriers for making cytotoxic-targeting complexes (Chapter 7).

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Preparation of Liposome Conjugates and Derivatives

A fast growing field that heavily depends on bioconjugate technology involves the use of liposomes. At one time, liposomes were studied only for their interesting structural characteristics in solution. Their physicochemical properties were investigated extensively as models of membrane morphology. Today, they are being put to use as macromolecular carriers for nearly every application of bioconjugate chemistry imaginable. They are used as delivery devices to encapsulate cosmetics, drugs, fluorescent detection reagents, and as vehicles to transport nucleic acids, peptides, and proteins to cellular sites *in vivo*. Targeting components such as antibodies can be attached to liposomal surfaces and used to create large antigen-specific complexes. In this sense, liposomal derivatives are being used to target cancer cells *in vivo*, to enhance detectability in immunoassay systems, and as multivalent cross-bridges in avidin–biotin-based assays. Covalent attachment of antigens to the surface of liposomes provides excellent immunogen complexes for the generation of specific antibodies or as vaccine carriers to elicit protective immunity.

The end-products of liposome technology are used in retail markets, for the diagnosis of disease, as therapeutic agents, as vaccines, and as important components in assays designed to either detect or quantify certain analytes.

The following sections discuss the properties and applications of liposome technology as well as the most common methods of preparing conjugates of them with proteins and other molecules.

1. Properties and Use of Liposomes

1.1. Liposome Morphology

Liposomes are artificial structures primarily composed of phospholipid bilayers exhibiting amphiphilic properties. Other molecules, such as cholesterol or fatty acids also may be included in the bilayer construction. In complex liposome morphologies, concentric spheres or sheets of lipid bilayers are usually separated by aqueous regions that are sequestered or compartmentalized from the surrounding solution. The phospholipid constituents of liposomes consist of hydrophobic lipid "tails" connected to a "head" constructed of various glycerylphosphate

derivatives. The hydrophobic interaction between the fatty acid tails is the primary driving force for creating liposomal bilayers in aqueous solution.

However, the organization of liposomes in aqueous solution may be highly complicated. The nature of the lipid constituents, the composition of the medium, and the temperature of the solution all affect the association and morphology of liposomal construction. Small "monomers" or groupings of lipid molecules may assemble to create larger structures having several main forms (Figure 22.1). Aggregation of these monomers may fuse them into spherical micelles, wherein the polar head groups are all facing outward toward the surrounding aqueous medium and the hydrophobic tails are all pointing inward, excluding water. In addition, aggregation may result in bilayer construction. In this case, sheets of lipid molecules, all with their head groups facing one direction and their tails facing the other way, are fused with another lipid sheet having their tails and heads facing the opposite direction. Thus, the inside of the bilayer contains only hydrophobic tails from both sheets, while the outside contains the hydrophilic heads facing the outer aqueous environment.

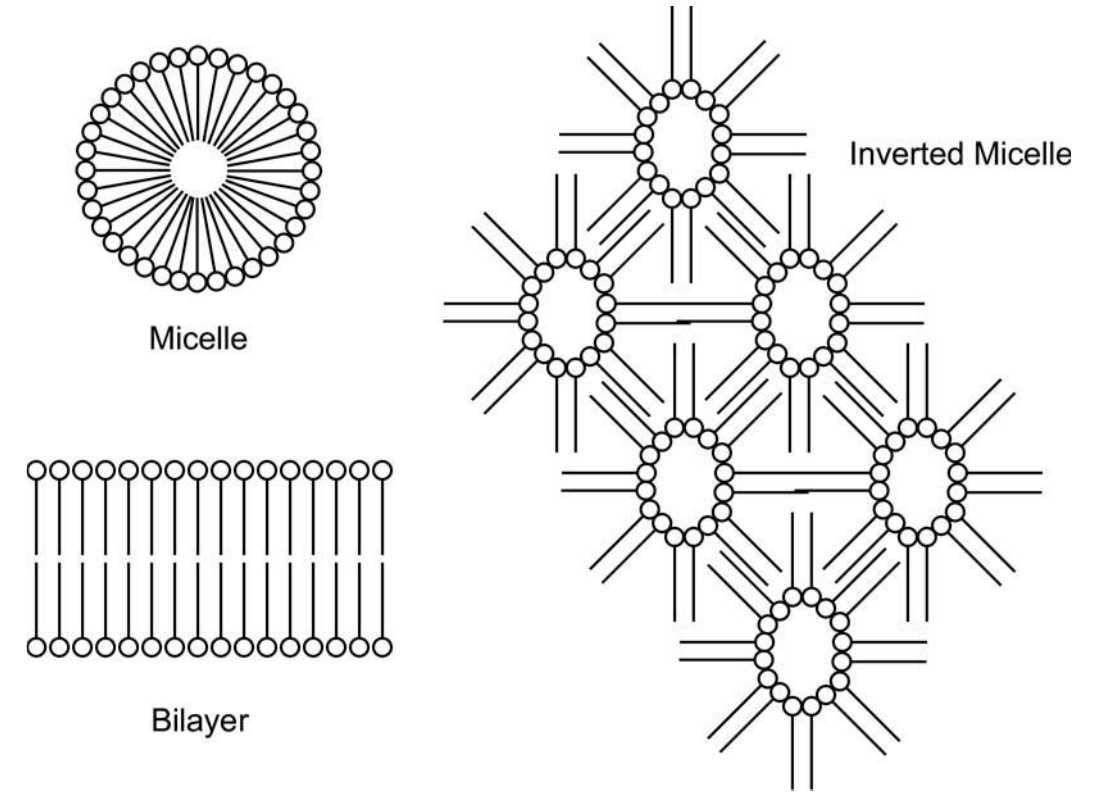


Figure 22.1 The amphiphilic nature of phospholipids in solution drives the formation of complex structures. Spherical micelles may form in aqueous solution, wherein the hydrophilic head groups all point out toward the surrounding water environment and the hydrophobic tails point inward to the exclusion of water. Larger lipid bilayers may form by similar forces, creating sheets, spheres, and other highly complex morphologies. In nonaqueous solution, inverted micelles may form, wherein the tails all point toward the outer hydrophobic region and the heads point inward forming hexagonal shapes.

The configuration the bilayers can assume also can be complex. A bilayer may be in a spherical form, having one layer of hydrophilic head groups pointing outward toward the surrounding solution and the second hydrophilic layer pointing inward toward a compartment of aqueous solution sequestered within the sphere. The morphology of a liposome may be classified according to the compartmentalization of aqueous regions between bilayer shells. If the aqueous regions are segregated by only one bilayer each, the liposomes are called unilamellar vesicles (ULV) (Figure 22.2). If there is more than one bilayer surrounding each aqueous compartment, the liposomes are termed multilamellar vesicles (MLV). ULV forms are further classified as to their relative size, although rather crudely. Thus, there can be small unilamellar vesicles (SUV; usually thought of as less than 100 nm in diameter, with a minimum of about 25 nm) and large unilamellar vesicles (LUV; usually greater than 100 nm in diameter, with a maximal size of about 2,500 nm). With regard to MLV, however, the bilayer structures cannot be as easily classified due

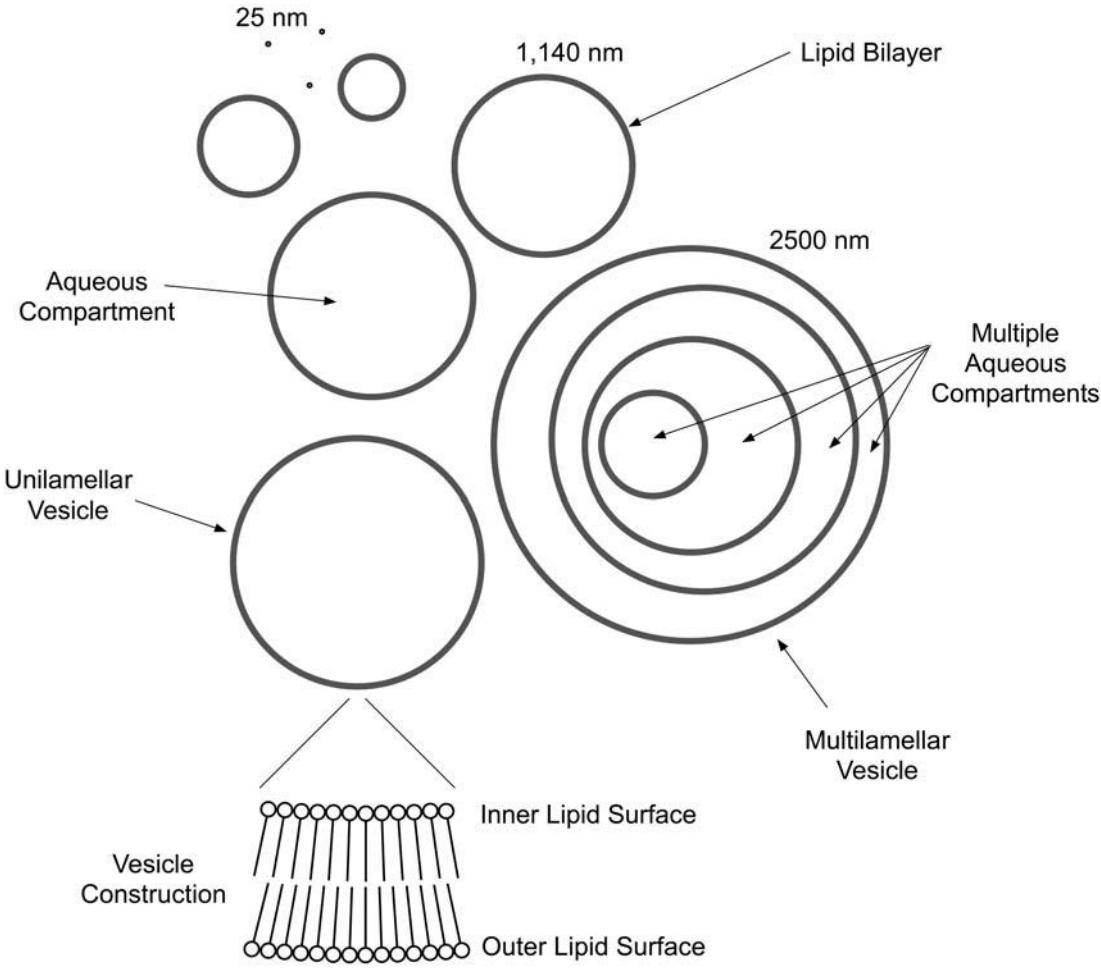


Figure 22.2 The highly varied morphologies of lipid bilayer construction.

to the almost infinite number of ways each bilayer sheet can be associated and interconnected with the next one. MLVs typically form large complex honeycomb structures that are difficult to categorize or exactly reproduce. However MLVs are the simplest to prepare, most stable, and easiest to scale up to large production levels.

Small lipid groupings or monomers also may fuse into bigger, inverted micelles, wherein their hydrophobic tails point outward toward other inverted micelle lipid tails. The individual structures are usually hexagonal in shape, but typically they exist as large groupings of inverted micelles, the outer edge of which contains partial inverted micelles, exposing their inner hydrophilic heads to the surrounding aqueous environment.

The most useful form of liposomes for bioconjugate applications consists of small, spherical ULVs that possess layers of hydrophilic head groups on their inner and outer surfaces. The inside of each vesicle can contain hydrophilic molecules that are protected from the outer environment by the lipid shell. The outside surface can be derivatized to contain covalently attached molecules designed to target the liposome for specific interactions.

1.2. Preparation of Liposomes

Mixtures of phospholipids in aqueous solution will spontaneously associate to form liposomal structures. To prepare liposomes having morphologies useful for bioconjugate or delivery techniques, it is necessary to control this assemblage to create vesicles of the proper size and shape. Many methods are available to accomplish this goal, however all of them have at least several steps in common: (1) dissolving the lipid mixture in organic solvent, (2) dispersion in an aqueous phase, and (3) fractionation to isolate the correct liposomal population.

In the first stage, the desired mix of lipid components is dissolved in organic solvent (usually chloroform:methanol (2:1 by volume)) to create a homogeneous mixture. This mixture will include any phospholipid derivatized to contain reactive groups as well as other lipids used to form and stabilize the bulk of the liposomal structure. During all handling procedures using lipids or their derivatives, it is essential that the solutions be protected from oxidation and excessive exposure to light, especially the sun. Organic solvents should be maintained under a nitrogen or argon atmosphere to prevent introduction of oxygen. Water and buffers should be degassed using a vacuum and bubbled with inert gas before introducing lipid components.

The correct ratio of lipid constituents is important to form stable liposomes. For instance, a reliable liposomal composition for encapsulating aqueous substances may contain molar ratios of lecithin:cholesterol:negatively charged phospholipid (e.g., phosphatidyl glycerol (PG)) of 0.9:1:0.1. A composition that is typical when an activated phosphatidylethanolamine (PE) derivative is included may contain molar ratios of phosphatidylcholine (PC):cholesterol:PG:derivatized PE of 8:10:1:1. Another typical composition using a maleimide derivative of PE without PG is PC:maleimide-PE:cholesterol of 85:15:50 (Friede *et al*., 1993). In general, to maintain membrane stability, the PE derivative should not exceed a concentration ratio of about 1–10 mol PE per 100 mol of total lipid.

An example of a lipid mixture preparation based on mass would be to dissolve 100 mg of PC, 40 mg of cholesterol, and 10 mg of PG in 5 ml of chloroform/methanol solution. When using activated PE components, inclusion of 10 mg of the PE derivative to this recipe will result in a stable liposome preparation.

Once the desired mixture of lipid components is dissolved and homogenized in organic solvent, one of several techniques may be used to disperse the liposomes in aqueous solution. These methods may be broadly classified as (1) mechanical dispersion, (2) detergent-assisted solubilization, and (3) solvent-mediated dispersion.

Probably the most popular option is mechanical dispersion, simply because the greatest number of methods that utilize it have been developed. When using mechanical means to form vesicles, the lipid solution first is dried to remove all traces of organic solvent prior to dispersion in an aqueous media. The dispersion process is the key to producing liposomal membranes of the correct morphology. This method uses mechanical energy to break up large lipid agglomerates into smaller vesicles having the optimal size and shape characteristics necessary for encapsulation or bioconjugation.

Mechanical dispersion methods involve adding an aqueous solution (which may contain substances to be encapsulated) to the dried, homogeneous lipid mixture and manipulating it to effect dispersion. Major methods of mechanical dispersion include simple shaking (Bangham *et al.*, 1965), non-shaken aqueous contact (Reeves and Dowben, 1969), high-pressure emulsification (Mayhew *et al.*, 1984), sonication (Huang, 1969), extrusion through small-pore membranes (Szoka *et al.*, 1980), and various freeze–thaw techniques (Pick, 1981). Some devices are available commercially which automate the mechanical dispersion process, usually by high-pressure emulsification or sonication (Branson Ultrasonics Corp.).

Most of these methods result in a population of vesicles ranging from SUVs of only 25 nm diameter to very large MLVs. Classification of the desired liposomal morphology may be done by chromatographic means using columns of Sepharose 2B or Sepharose 4B, by density-gradient centrifugation using Ficoll or metrizamide gradients, or by dialysis.

Liposome formation by detergent-assisted solubilization utilizes the amphipathic nature of detergent molecules to bring more effectively the lipid components into the aqueous phase for dispersion. The detergent molecules presumably bind and mask the hydrophobic tails of lipids from the surrounding water molecules. Detergent treatment may take place from a dried lipid mixture or after formation of small vesicles. Usually, nonionic detergents such as the Triton X family, alkyl glycosides, or bile salts such as sodium deoxycholate are employed for this procedure. The immediate structures which form as the detergent molecules solubilize the lipids from a dried state are small micelles. Upon removal of the detergent from the solution, the lipid micelles aggregate to create larger liposome structures. Liposomes of up to 1,000 Å containing a single bilayer may be formed using detergent-assisted methods (Enoch and Strittmatter, 1979). Unfortunately, some detergent-removal processes also may remove other molecules that were to be entrapped in the liposomes during formation.

Solvent-mediated dispersion techniques used to create liposomes first involve dissolving the lipid mixture in an organic solvent to create a homogeneous solution, and then introducing this solution into an aqueous phase. The solvent may or may not be soluble in the aqueous phase to effect this process. There also may be components dissolved in the aqueous phase to be encapsulated in the developing liposomes.

Perhaps the simplest solvent dispersion method is that developed by Batzri and Korn (1973). Phospholipids and other lipids to be a part of the liposomal membrane are first dissolved in ethanol. This ethanolic solution then is rapidly injected into an aqueous solution of 0.16 M KCl using a syringe, resulting in a maximum concentration of no more than 7.5 percent ethanol. Using this method, single bilayer liposomes of about 25 nm diameter can be created that are

indistinguishable from those formed by mechanical sonication techniques. The main disadvantages of ethanolic injection are the limited solubility of some lipids in the solvent (about 40 mM for PC) and the dilute nature of the resultant liposome suspension. However, for the preparation of small quantities of SUVs, this method may be one of the best available.

Other solvent dispersion methods utilize solvents that are insoluble in the aqueous phase. The key to the production of liposomes by this procedure involves the formation of a "water-in-oil" emulsion. To create the proper reverse-phase emulsion, a small quantity of aqueous phase must be introduced into a large quantity of organic phase containing the dissolved liposomes. The result is a milky dispersion containing the "homogenized" liposomes. A number of techniques have been developed to perform this procedure (Kim and Martin, 1981; Kim *et al*., 1983; Pidgeon *et al*., 1986). The emulsification process in each of these solvent-dispersion techniques involves the use of mechanical means (shaking, stirring, or sonication) to effect the formation of small droplets of aqueous solution uniformly dispersed in the lipid–organic phase.

For the preparation of large quantities of liposomes, mechanical dispersion using a commercially available emulsifier is probably the best route. For limited quantities, the use of simple shaking or ethanolic dispersion techniques works well.

Regardless of their method of fabrication, most liposome preparations need to be further classified and purified before use. To remove excess aqueous components that were not encapsulated during the vesicle formation process, gel filtration using a column of Sephadex G-50 or dialysis can be employed. To fractionate the liposome population according to size, gel filtration using a column of Sepharose 2B or 4B should be done.

Small liposome vesicles often aggregate upon standing to form larger, more complex structures. Therefore, long-term storage in aqueous solution is usually not possible without major transformations in liposome morphology. Freezing also fractures the liposomal membrane, releasing any entrapped substances. The inclusion of cryoprotectants such as sugars or polyhydroxylic-containing compounds can overcome the structural degradation problems upon freezing (Harrigan *et al*., 1990; Talsma *et al*., 1991; Park and Huang, 1992). Presumably, the hydroxyl groups in cryoprotectants can take the place of water in hydrogen bonding activities, thus providing structural support even under conditions in which water is removed. A procedure by Friede *et al*. (1993) allows freezing and lyophilization of SUVs in the presence of 4 percent sorbitol with complete retention of liposome integrity upon reconstitution. Thus freeze-drying may be the best method for the long-term storage of intact liposomes.

1.3. Chemical Constituents of Liposomes

The overall composition of a liposome—its morphology, chemical constituents (including a large variety of phospholipids and other lipids or fatty acids), charge, and any attached functional groups—can affect the properties of the vesicle both *in vitro* and *in vivo* (Allison and Gregoriadis, 1974; Alving, 1987; Therien and Shahum, 1989). Although there are literally dozens of lipid components that potentially can be included in a liposomal recipe, only a handful are commonly used.

Phospholipids are the most important of these liposomal constituents. Being the major component of cell membranes, phospholipids are composed of a hydrophobic, fatty acid tail, and a hydrophilic head group. The amphipathic nature of these molecules is the primary force that drives the spontaneous formation of bilayers in aqueous solution and holds the vesicles together.

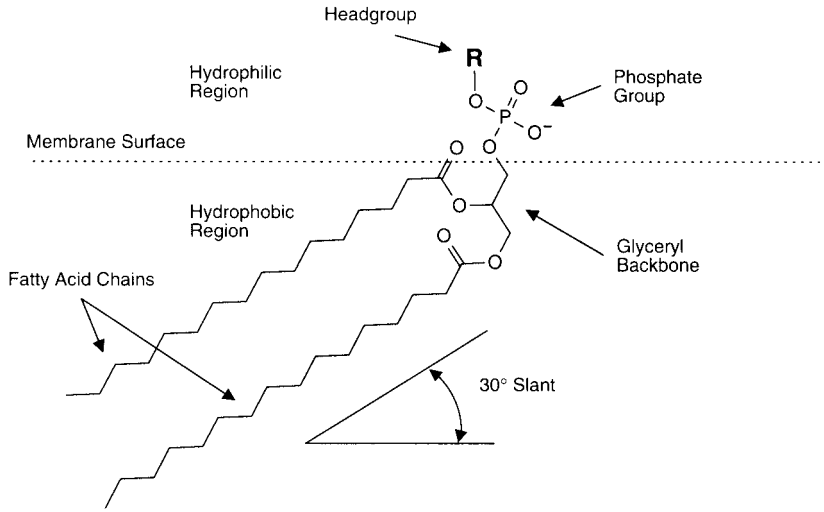


Figure 22.3 The basic construction of phosphodiglyceride molecules within lipid bilayers. The fatty acid chains are embedded in the hydrophobic inner region of the membrane, oriented at an angle to the plane of the membrane surface. The hydrophilic head group, including the phosphate portion, points out toward the hydrophilic aqueous environment.

Naturally occurring phospholipids can be isolated from a variety of sources. One of the most common phospholipid raw materials is egg yolk. However, since the composition of egg phospholipid is from a biological source and can vary considerably depending on age of the eggs, the diet of the chickens, and the method of processing, newer enzymatic and synthetic chemical methods now are being employed to manufacture the required phospholipid derivatives in higher purity and yield.

Two main forms of lipid derivatives exist biologically: molecules containing a glycerol backbone and those containing a sphingosine backbone. The most important type for liposomal construction is a phosphodiglyceride derivative, which consists of a glycerol backbone that links two fatty acid molecules with a polar head group (Figure 22.3). The fatty acids are acyl bonded in ester linkages to the Nos. 1 and 2 carbon hydroxyls of the glycerol bridge. The No. 3 carbon hydroxyl of the glycerol group is phosphorylated and possesses a negative charge at physiological pH. This basic phosphodiglyceride construct of two fatty acids and one glycerylphosphate group is called phosphatidic acid. This is the simplest form of phospholipid available.

The fundamental phosphatidyl group also can be further derivatized at the phosphate to contain an additional polar constituent. Several common derivatives of phosphodiglycerides are naturally occurring, including PC; commonly called lecithin, phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl glycerol (PG), and phosphatidyl inositol (PI) (Figure 22.4). All of these phospholipids have polar groups that are linked to the phosphatidyl moiety in a phosphate ester bond. The most abundant of these derivatives in biological cell membranes is PC—the trimethyl derivative of PE, possessing a positive charge at physiological pH. Some or all of these phosphodiglyceride derivatives can be mixed to create a particular liposomal recipe.

The fatty acid constituents of phosphodiglycerides can vary considerably in nature among a number of different chain lengths and points of unsaturation. A given isolated phosphatidyl

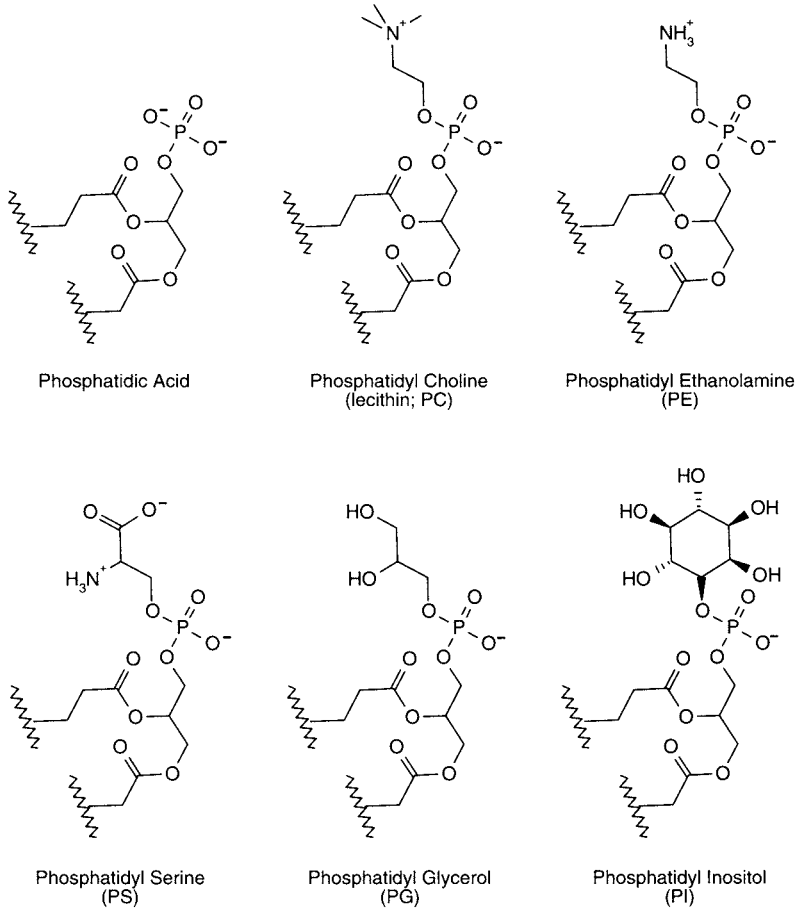


Figure 22.4 The head-group construction of the six commonly encountered phosphatidyl derivatives.

derivative from a biological source usually possesses a range of fatty acid components, varying in chain length from C16 to about C24. Some of the fatty acids also may contain points of unsaturation—one or more double bonds between certain carbon atoms within the chain (Matreya and Avanti Polar Lipids, suppliers). For instance, egg lecithin is not a single compound, but contains a mixture of PC containing about 31 percent saturated fatty acid having a chain length of 16 carbons, 16 percent saturated fatty acid with 18 carbons, about 48 percent also with 18 carbons but having at least 1–2 points of unsaturation, and the rest a variety of other fatty acid constituents. Naturally occurring, unsaturated fatty acids typically are of the *cis* conformation, not *trans*. The existence of unsaturation within a fatty acid is usually abbreviated as the chain length followed by a colon and the number of double bonds. For instance, *cis*-9-hexadecenoic acid (palmitoleic acid) contains one double bond at carbon 9 and it is abbreviated as C16:1.

By contrast, a given synthetic preparation of a major phospholipid possesses fatty acid constituents all of identical chain length and unsaturation. A synthetic PC derivative can be purchased that contains only, for instance, 1,2-dimyristoyl (C14) fatty acid substitutions on its glyceryl

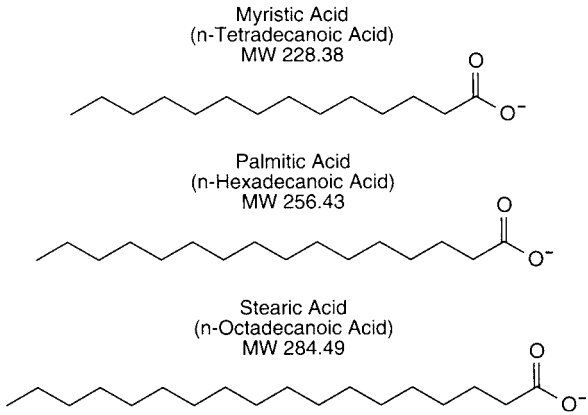


Figure 22.5 The three fatty acid components commonly used in liposome construction.

backbone (Genzyme). The use of synthetic rather than natural phospholipids for making liposomes thus produces reagents of known chemical purity, which is very important for regulatory requirements surrounding the introduction of products used topically or *in vivo*.

Despite the large variety of potential fatty acid components in natural-occurring phosphodiglycerides, only three major fatty acid derivatives of synthetic phospholipids are commonly used in liposome preparation: (1) myristic acid (*n*-tetradecanoic acid; containing 14 carbons), (2) palmitic acid (*n*-hexadecanoic acid; containing 16 carbons), and (3) stearic acid (*n*-octadecanoic acid; containing 18 carbons) (Figure 22.5).

The nomenclature for associating individual fatty acid groups with particular phosphodiglyceride derivatives is straightforward. For instance, a phosphatidic acid (PA) derivative which contains two myristic acid chains is commonly called dimyristoyl phosphatidic acid (DMPA). Likewise, a PC derivative containing two palmitate chains is called dipalmitoyl phosphatidyl choline (DPPC). Other phosphodiglyceride derivatives are similarly named.

The second form of lipid derivative that occurs naturally in membrane structures is derived from sphingosine. Unlike the phosphodiglyceride derivatives discussed above, sphingolipids contain no glycerol backbone. Instead, these lipids are constructed from a derivative of 4-sphingenine, containing an *N*-acyl-linked fatty acid group and possibly other constituents off the No. 1 carbon hydroxyl group (Figure 22.6). Sphingolipids are highly similar in their construction to glyceryl lipids, in that there are two hydrophobic tails present on a 3-carbon backbone (one of them contributed from 4-sphingenine itself and the other from the attached fatty acid), and there also exists a hydrophilic head group. This creates the typical amphipathic properties common to all lipid membrane components.

The simplest form of sphingolipid, ceramide, contains a fatty acid group, but no additional components on the No. 1 hydroxyl. Major derivatives of ceramide at the 1-hydroxyl position include a positively charged phosphocholine compound, called sphingomyelin, a glucose derivative, called glucosylcerebroside, and other complex carbohydrate derivatives, termed gangliosides (Figure 22.7). Gangliosides are involved in various cellular recognition phenomena, including being part of the blood group determinants, A, B, and O, in humans.

The use of sphingolipids in liposome formation is possible due to the natural amphipathic properties of the molecules. Some sphingolipids can lend structural advantages to the integrity

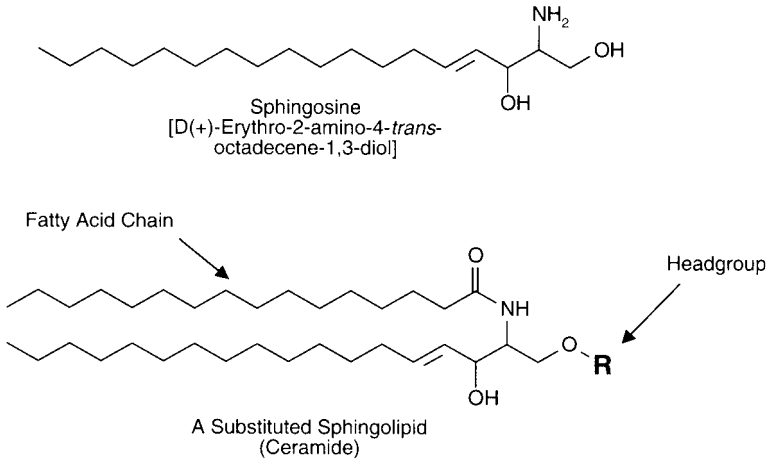


Figure 22.6 Sphingolipids are constructed of sphingosine derivatives containing an acylated fatty acid and a head group attached to the hydroxyl.

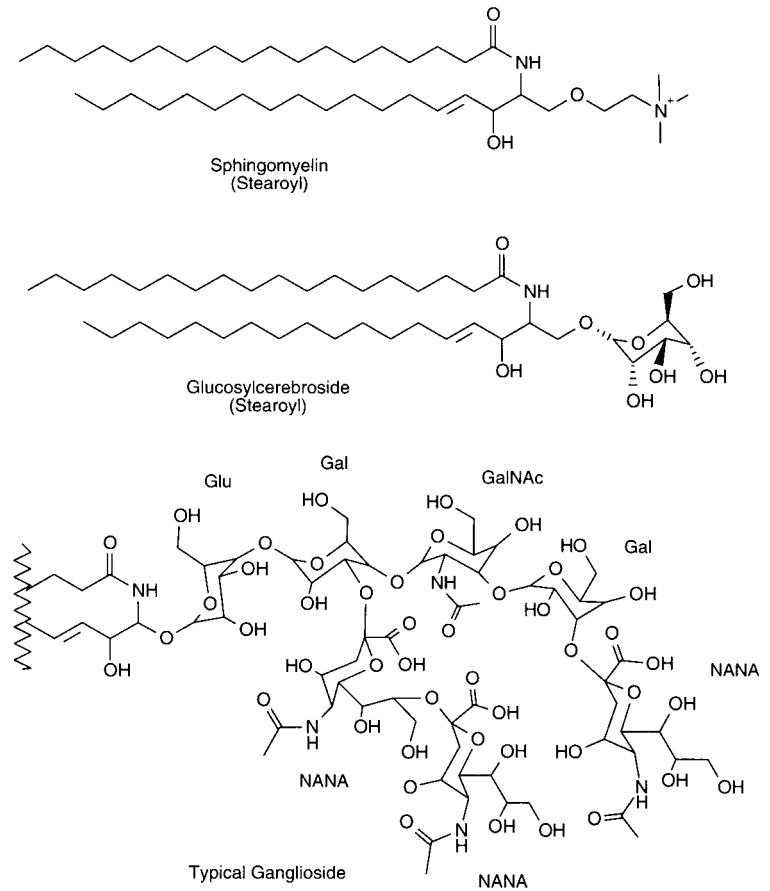


Figure 22.7 Common sphingolipid derivatives include small and highly complex head groups.

of liposomal membranes. Sphingomyelin, for example, is capable of hydrogen bonding with adjacent glyceryl lipids, thus increasing the order and stability of the vesicle construction. This stability may translate into a lower potential for passage of molecules through the membrane bilayer, forming vesicles that are better able to retain their contents than more fluid liposome constructions. The temperature of phase transition in sphingolipid-containing membranes is often greater than membranes constructed of only phosphodiglyceride derivatives. Liposomes containing sphingomyelin or gangliosides also have prolonged lifetimes *in vivo* (Gregoriadis and Senior, 1980; Allen and Chonn, 1987) and may be advantageous for creating liposome immunogen complexes.

The main disadvantage of incorporating sphingolipids in liposomes is their high cost. Purified phosphodiglyceride derivatives may be obtained in bulk quantities and in highly defined synthetic preparations, whereas sphingolipid derivatives are not so readily available in similar purity.

Another significant component of many liposome preparations is cholesterol. In natural cell membranes, cholesterol makes up about 10–50 percent of the total lipid. For liposome preparation, it is typical to include a mole ratio of about 50 percent cholesterol in the total lipid recipe. The addition of cholesterol to phospholipid bilayers alters the properties of the resultant membrane in important ways. As it dissolves in the membrane, cholesterol orients itself with its polar hydroxyl group pointed toward the aqueous outer environment, approximately even, in a three-dimensional sense, with the glyceryl backbone of the bilayer's phosphodiglyceride components (Figure 22.8). Structurally, cholesterol is a rigid component in membrane construction, not having the same freedom of movement that the fatty acid tails of phosphodiglycerides possess. Adjacent phospholipid molecules are restricted in their freedom of movement throughout the length of their fatty acid chains that are abutting the cholesterol molecules. However, since the cholesterol components have the effect of creating spaces in the uniform hydrophobic morphology of the bilayer, the portion of the fatty acid chains below the abutted regions are increased in their freedom of movement.

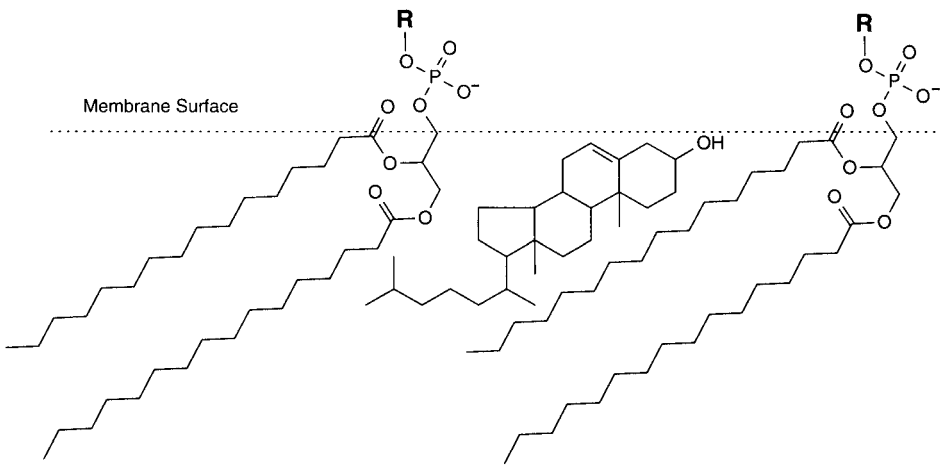


Figure 22.8 The orientation of cholesterol in phospholipid bilayers.

Cholesterol's presence in liposome membranes has the effect of decreasing or even abolishing (at high cholesterol concentrations) the phase transition from the gel state to the fluid or liquid crystal state that occurs with increasing temperature. It also can modulate the permeability and fluidity of the associated membrane—increasing both parameters at temperatures below the phase transition point and decreasing both above the phase transition temperature. Most liposomal recipes include cholesterol as an integral component in membrane construction.

1.4. Functional Groups of Phospholipids

For the production of liposomal conjugates, lipid derivatives must be incorporated into the bilayer construction that contain available functional groups able to be chemically crosslinked or modified. A number of phosphodiglyceride compounds can be employed for conjugation purposes. Each of these components contains a head group that can be directly derivatized or chemically modified to contain a reactive group. For instance, several lipid derivatives contain amine groups that can be utilized in nucleophilic reactions with crosslinkers or other modification reagents. These include PE, PS, and stearylamine. Carboxyl-containing molecules include all the individual fatty acids as well as PS. These can be coupled to amine-containing molecules by the use of the carbodiimide reaction (Chapter 3, Section 1). Hydroxyl-containing lipids include PG, fatty acid alcohols, PI, and various gangliosides and cerebrosides of sphingolipid derivation. Lipids possessing hydroxyl groups on adjacent carbon atoms, such as those containing sugar constituents, may be oxidized with sodium periodate to produce reactive aldehyde residues (Chapter 1, Section 4.4). Coupling aldehydes to amine-containing molecules can be accomplished by reductive amination (Chapter 3, Section 4). Finally, the phosphate groups of PA residues may be used to conjugate with amine-containing molecules in a method similar to modification of the 5′-phosphates of DNA probes. This is done through the use of the carbodiimide reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Chapter 27, Section 2.1). Figure 22.9 shows the structures and reactive sites of these lipid functional groups.

When liposomes are used as part of a conjugate system, the targeting molecules usually are attached covalently to these head group functional groups using standard crosslinking chemistries (Derksen and Scherphof, 1985). Although all of the above-mentioned functional groups on lipid molecules can be used for the conjugation process, most often the derivatization reaction is done off the PE constituents within the liposomal mixture. The primary amine modification off the glycerylphosphate head of PE provides an ideal functional group for activation and subsequent coupling of targeting or detection molecules (Shek and Heath, 1983). Liposomes may be constructed with reactive groups already prepared on their PE constituents, all set to be conjugated with selected molecules having the correct functional group. Stock preparations of activated liposomes may even be prepared and lyophilized to be used as needed in coupling macromolecules (Friede *et al.*, 1993). All of the amine-reactive conjugation methods discussed in this section may be used with PE-containing liposomes.

2. Derivatization and Activation of Lipid Components

Two approaches for the activation of lipid components may be used to create reactive groups in liposomes. A purified lipid may be activated prior to incorporation into the bilayer construction

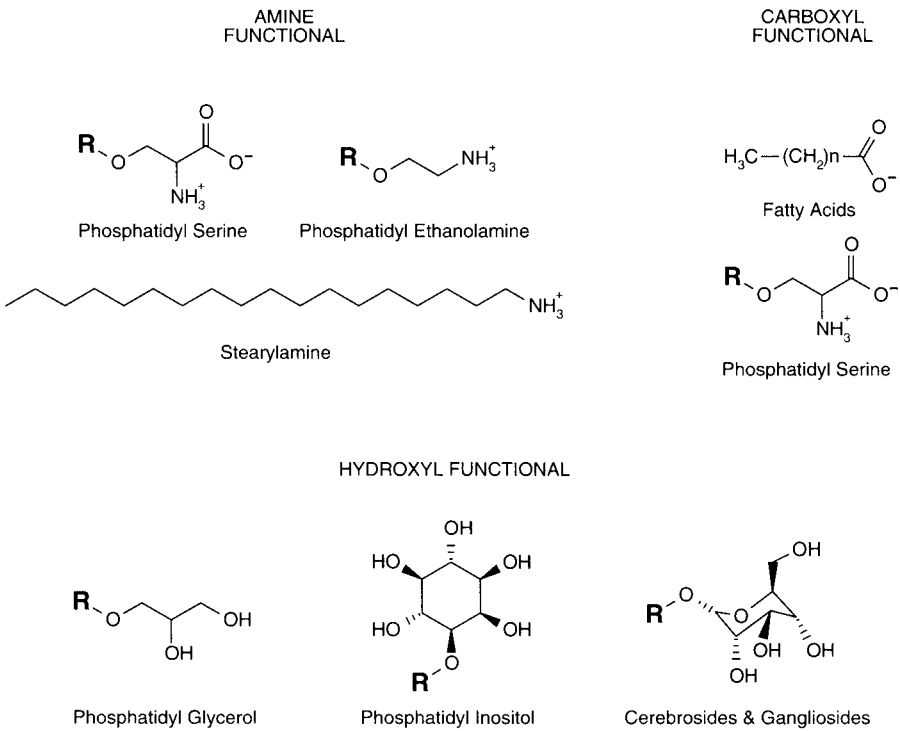


Figure 22.9 The major functional groups of lipids that may participate in bioconjugate techniques include amines, carboxylates, and hydroxyls.

or the activation step may occur after formation of the intact liposome. Either way, the goal of the activation process is to provide a reactive species that can be used to couple with selected target groups on proteins or other molecules. While numerous crosslinking methods can be used with lipid functional groups, three main strategies commonly are used to conjugate proteins with liposomes: (1) reductive amination to couple aldehyde residues with amines, (2) carbodiimide-mediated coupling of an amine to a carboxylate or an amine to a phosphate group, and (3) multi-step, heterobifunctional crosslinker-mediated conjugation. Both reductive amination and heterobifunctional processes involve activation of particular lipid components.

2.1. Periodate Oxidation of Liposome Components

Reductive amination-mediated conjugation can be done by periodate-oxidizing carbohydrate or glycerol groups on lipid components and using them to couple with amine-containing molecules. It also may be accomplished by using amines on liposomes (i.e., by the incorporation of PE or SA residues) and coupling them to aldehydes present on proteins or other molecules. Using the first approach, liposomes containing PG or glycosphingolipid residues are oxidized by sodium periodate, purified, and then used to conjugate with protein molecules in the presence of sodium cyanoborohydride (Figure 22.10) (Chapter 3, Section 4). A protocol for the formation of aldehyde groups on liposomes can be found in the method of Heath *et al.* (1981).

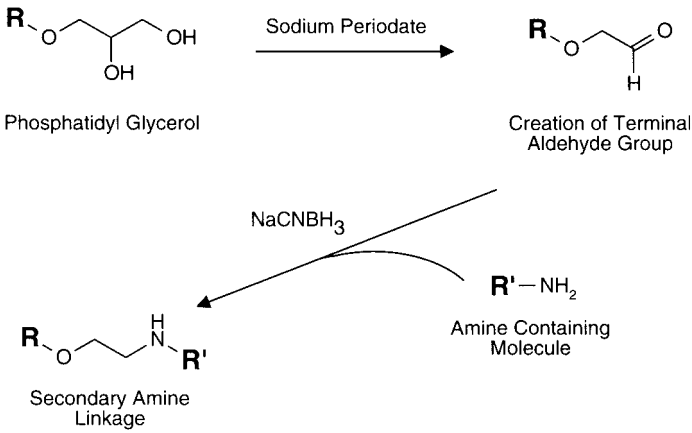


Figure 22.10 Hydroxylic-containing lipid components, such as PG, may be oxidized with sodium periodate to produce aldehyde residues. Modification with amine-containing molecules then may take place using reductive amination.

Protocol

1. Prepare a 5 mg/ml liposome construction in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4, containing, on a mole ratio basis, a mixture of PC:cholesterol:PG:other glycolipids of 8:10:1:2. The other glycolipids that can be incorporated include PI, lactosylceramide, galactose cerebroside, or various gangliosides. Other liposome compositions may be used, for example recipes without cholesterol, as long as a periodate-oxidizable component containing diols is present. Any method of liposome formation may be used.
2. Dissolve sodium periodate to a concentration of 0.6 M by adding 128 mg per ml of water. Add 200 µl of this stock periodate solution to each ml of the liposome suspension with stirring.
3. React for 30 minutes at room temperature in the dark.
4. Dialyze the oxidized liposomes against 20 mM sodium borate, 0.15 M NaCl, pH 8.4, to remove unreacted periodate. This buffer is ideal for the subsequent coupling reaction. Chromatographic purification using a column of Sephadex G-50 also can be done.

The periodate-oxidized liposomes may be used immediately to couple with amine-containing molecules such as proteins (see Section 7.6), or they may be stored in a lyophilized state in the presence of sorbitol (Friede *et al.*, 1993) for latter use.

2.2. Activation of PE Residues with Heterobifunctional Crosslinkers

The most common type of heterobifunctional reagent used for the activation of lipid components includes the amine- and sulfhydryl-reactive crosslinkers containing an *N*-hydroxysuccinimide (NHS) ester group on one end and either a maleimide, iodoacetyl, or pyridyl disulfide group on the other end (Chapter 5, Section 1). Principle reagents used to effect this activation process include SMCC (Chapter 5, Section 1.3), MBS (Chapter 5, Section 1.4), SMPB (Chapter 5, Section 1.6), SIAB (Chapter 5, Section 1.5), and SPDP (Chapter 5, Section 1.1). Other

hydrophilic heterobifunctional crosslinkers containing a discrete polyethylene glycol (PEG) spacer may be used in a similar manner (Chapter 18, Section 2).

Activation of PE residues with these crosslinkers can proceed by one of two routes: the purified PE phospholipid may be modified in organic solvent prior to incorporation into a liposome, or an intact liposome containing PE may be activated while suspended in aqueous solution. Most often, the PE derivative is prepared before the liposome is constructed. In this way, a stable, stock preparation of modified PE may be made and used in a number of different liposomal recipes to determine the best formulation for the intended application. However, it may be desirable to modify PE after formation of the liposomal structures to ensure that only the outer half of the lipid bilayer is altered. This may be particularly important if substances to be entrapped within the liposome are sensitive or react with the PE derivatives.

Crosslinkers used to activate PE should be of the longest spacer variety available. The length of the spacer is important in providing enough distance from the liposome surface to accommodate the binding of another macromolecule. Short activating reagents often restrict protein accessibility to approach close enough to react with the functional groups on the bilayer surface. For instance, direct modification of PE with iodoacetate results in little or no sulfhydryl-modified IgG coupled to the associated liposomes. When an aminoethylthioacetyl spacer is used to move the iodoacetyl group farther away from the bilayer surface, good IgG coupling occurs (Hashimoto *et al*., 1986). The use of longer discrete PEG-based crosslinkers may enhance the coupling of proteins to liposome surfaces, because the extreme water-solubility of the spacer provides greater aqueous phase access to approaching proteins (Chapter 18, Section 2). However, this concept does not apply to the coupling of low-molecular-weight molecules that can access the surface chemistry more readily than macromolecules.

For the activation of PE prior to liposome formation, it is best to employ a highly purified form of the molecule. While egg PE is abundantly available, it consists of a range of fatty acid derivatives—many of which are unsaturated—and is highly susceptible to oxidation. Synthetic PE, by contrast, can be obtained having a discrete fatty acid composition and is much more stable to oxidative degradation.

The following suggested protocols are modifications of those described by Martin and Papahadjopoulos (1982), Martin *et al*. (1990), and Hutchinson *et al*. (1989). Although the methods were developed for use with SMPB, SPDP, and MBS, the same basic principles can be used to activate PE with any of the heterobifunctional crosslinkers mentioned above. In addition, the use of hydrophilic NHS-PEG$_n$-maleimide compounds (Chapter 18) may be a superior alternative to the use of crosslinkers with hydrophobic cross-bridges, as the PEG linkers won't dissolve within the lipid bilayer structure. The reaction sequence for activation and coupling using SMPB is shown in Figure 22.11. The PE employed should be of a synthetic variety having fatty acid constituents of either dimyristoyl (DMPE), dipalmitoyl (DPPE), or distearoyl (DSPE) forms. For activation of pure PE, the heterobifunctional reagents should not be of the sulfo-NHS variety, since they are best used in aqueous reaction mediums and PE is activated under nonaqueous conditions. For activation of intact liposomes in aqueous suspension, the sulfo-NHS variety of the crosslinkers may be the best choice, since they are incapable of penetrating membranes, and thus only the outer surfaces of vesicles will be modified.

Protocol for the Activation of DPPE with SMPB

1. Dissolve 100 µmol of PE in 5 ml of argon-purged, anhydrous methanol containing 100 µmol of triethylamine (TEA). Maintain the solution over an argon or nitrogen

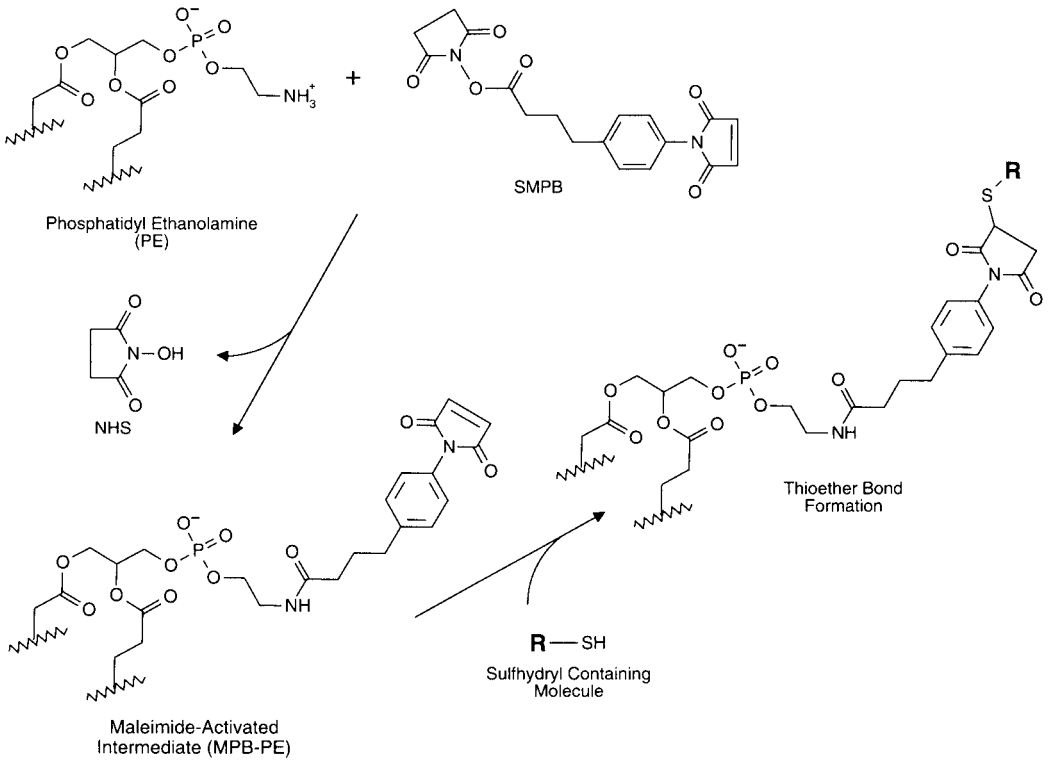


Figure 22.11 A sulfhydryl-reactive lipid derivative may be prepared through the reaction of SMPB with PE to produce a maleimide-containing intermediate. Sulfhydryl-containing molecules then may be coupled to the phospholipid via stable thioether linkages.

atmosphere. The reaction also may be done in dry chloroform. *Note*: Methanol or chloroform and TEA should be handled in a fume hood.

2. Add 50 mg of SMPB (Thermo Fisher) to the PE solution. Mix well to dissolve.
3. React for 2 hours at room temperature, while maintaining the solution under an argon or nitrogen atmosphere. Reaction progress may be determined by thin-layer chromatography (TLC) using silica gel 60-F_{254} plates (Merck) and developed with a 65:25:4 (by volume) mixture of chloroform:methanol:water. The activated PE derivative will develop faster on TLC (R_f = 0.52 for MPB–PE) than the unmodified PE.
4. Remove the methanol from the reaction solution by rotary evaporation and redissolve the solids in chloroform (5 ml).
5. Extract the water-soluble reaction by-products from the chloroform with an equal volume of 1 percent NaCl. Extract twice.
6. Purify the MPB–PE derivative by chromatography on a column of silicic acid (Martin *et al.*, 1981). The following description is from Martin *et al.*, 1990. Add 2 g silicic acid to 10 ml of chloroform and pour the solution into a syringe barrel containing a plug of glass wool at the bottom. Apply the chloroform-dissolved lipids on the silicic acid column. Wash with 4 ml of chloroform, then elute with 4 ml each of the following series of chloroform:methanol

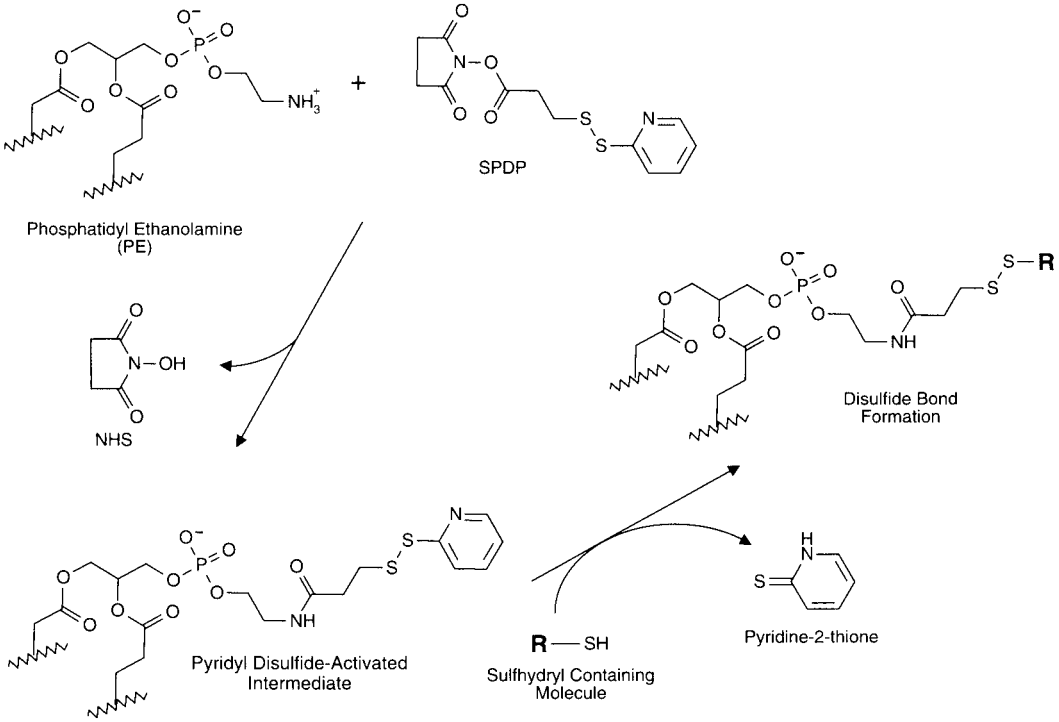


Figure 22.12 The reaction of SPDP with PE creates a maleimide derivative capable of coupling thiols. Reaction with a sulfhydryl-containing molecule forms a conjugate through a thioether linkage.

mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. During the chromatography, collect 2 ml fractions. Monitor for the presence of purified MPB–PE by TLC according to step 3.

7. Remove chloroform from the MBP–PE by rotary evaporation. Store the derivative at −20°C under a nitrogen atmosphere until use.

N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) also may be used to activate pure PE lipids in a similar manner to SMPB. The result will be a derivative containing pyridyl disulfide groups rather than maleimide groups (Figure 22.12). Pyridyl disulfides react with sulfhydryls to form disulfide linkages. Either the standard SPDP or the long-chain version, LC-SPDP, may be employed in the following protocol.

Protocol for Activation of PE with SPDP

1. Dissolve 20 μmol of PE (15 mg) in 2 ml of argon-purged, anhydrous methanol containing 20 μmol of triethylamine (2 mg). Maintain the solution over an argon or nitrogen atmosphere. The reaction also may be done in dry chloroform. *Note*: Methanol or chloroform and TEA should be handled in a fume hood.
2. Add 30 μmol (10 mg) of SPDP (Thermo Fisher) to the PE solution. Mix well to dissolve.
3. React for 2 hours at room temperature, while maintaining the solution under an argon atmosphere. Reaction progress may be determined by TLC using silica gel plates developed

with a 65:25:4 (by volume) mixture of chloroform:methanol:water. The activated PE derivative (PDP-PE) will develop faster on TLC than the unmodified PE.
4. Remove the methanol from the reaction solution by rotary evaporation and re-dissolve the solids in chloroform (5 ml).
5. Extract the water-soluble reaction by-products from the chloroform with an equal volume of 1 percent NaCl. Extract twice.
6. Purify the PDP–PE derivative by chromatography on a column of silicic acid (Martin *et al.*, 1981). The following description is from Martin *et al.* (1990). Add 2 g silicic acid to 10 ml of chloroform and pour the solution into a syringe barrel containing a plug of glass wool at the bottom. Apply the chloroform-dissolved lipids on the silicic acid column. Wash with 4 ml of chloroform, then elute with 4 ml each of the following series of chloroform:methanol mixtures: 4:0.25, 4:0.5, 4:0.75, and 4:1. During the chromatography, collect 2 ml fractions. Monitor for the presence of purified PDP–PE by TLC according to step 3.
7. Remove chloroform from the PDP–PE by rotary evaporation. Store the derivative at −20°C under a nitrogen atmosphere until use.

Other heterobifunctional reagents containing an NHS ester end can be used to activate PE in a similar manner to those protocols described above. A somewhat abbreviated protocol (eliminating the silicic acid chromatography step) for the activation of DPPE with MBS (Chapter 5, Section 1.4), as adapted from Hutchinson *et al.* (1989), follows. The NHS ester of MBS reacts with PE's free amine group to create an amide bond. The maleimide end of the crosslinker then remains available for subsequent conjugation with a sulfhydryl-containing molecule after liposome formation (Figure 22.13).

Protocol for the Activation of DPPE with MBS

1. Dissolve 40 mg of DPPE in a mixture of 16 ml dry chloroform and 2 ml dry methanol containing 20 mg TEA. Maintain under nitrogen to prevent lipid oxidation.
2. Add 20 mg of MBS to the lipid solution and mix to dissolve.
3. React for 24 hours at room temperature under nitrogen.
4. Wash the organic phase 3 times with PBS, pH 7.3, to extract excess crosslinker and reaction by-products.
5. Remove the organic solvents by rotary evaporation under vacuum.
6. Analyze the MBS–DPPE derivative by TLC using a silica plate and developing with a solvent mix containing chloroform:methanol:glacial acetic acid in the volume ratio of 65:25:13. The R_f value of underivatized DPPE is 0.56, while that of the MBS–DPPE product is R_f 0.78.

The MBS–DPPE derivative can be stored dry under a nitrogen blanket at 4°C or dissolved in chloroform:methanol (9:1, v/v) under the same conditions.

If intact liposomes containing PE are to be activated with these crosslinkers, the methods employed are similar to those used to modify proteins and other macromolecules in aqueous solution. The following protocol is a generalized version for the activation of liposomes containing PE with SPDP (Figure 22.14).

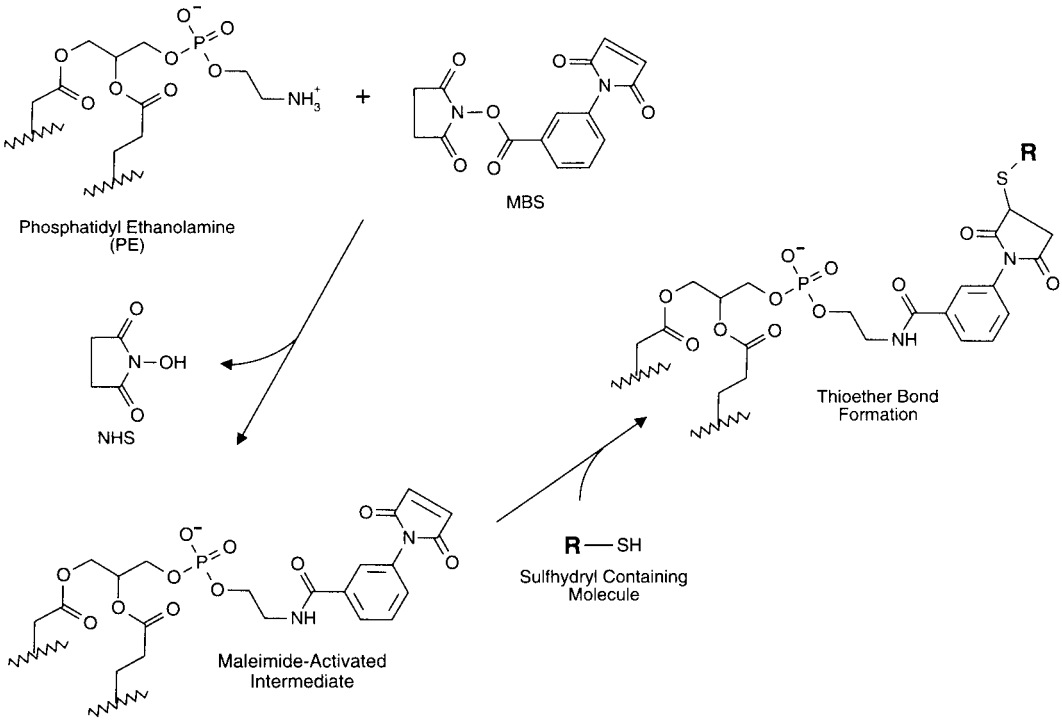


Figure 22.13 MBS reacted with PE produces a maleimide derivative that can couple to thiol compounds through a stable thioether bond.

Protocol for the Activation of Liposomes with SPDP

1. Prepare a 5 mg/ml liposome suspension in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.5, containing, for example, a mixture of PC:cholesterol:PG:PE at molar ratios of 8:10:1:1. Other lipid recipes may be used as long as they contain about this percent of PE. In addition, if this level of cholesterol is maintained in the liposome, then the integrity of the bilayer will be stable up to a level of organic solvent addition of about 5 percent. This factor is important for adding an aliquot of the crosslinker to the liposome suspension as a concentrated stock solution in an organic solvent. Dispersion of the liposomes to the desired size and morphology may be done by any common method (see Section 1.2, this chapter).
2. Dissolve SPDP (Thermo Fisher) at a concentration of 6.2 mg/ml in dimethylformamide (DMF) (makes a 20 mM stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMF (also makes a 20 mM solution). If the water-soluble sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to adding an aliquot to the reaction. The sulfo-NHS form of the crosslinker contains a negatively charged sulfonate group which prevents the reagent from penetrating lipid bilayers. Thus, only the outer surfaces of the liposomes can be activated using sulfo-LC-SPDP. If this is desirable, prepare a 10 mM solution of sulfo-LC-SPDP by dissolving

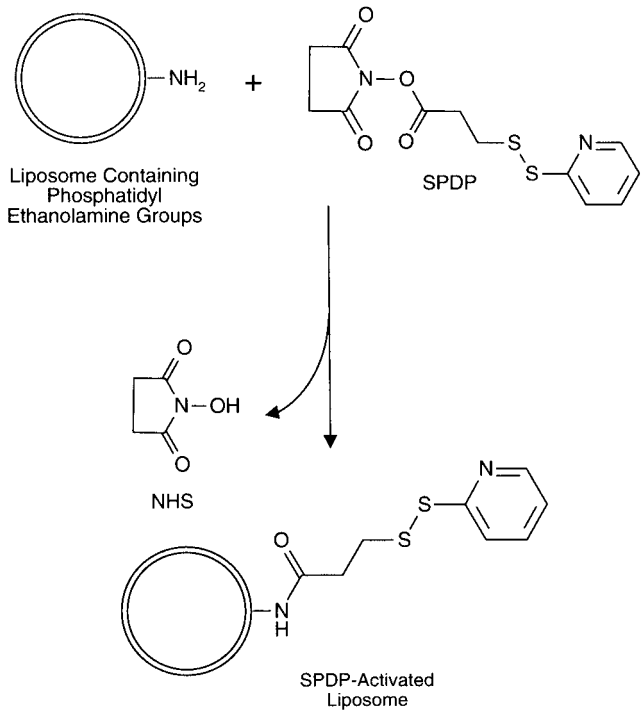


Figure 22.14 Intact liposomes containing PE components may be modified with SPDP to produce thiol-reactive derivatives.

5.2 mg/ml in water. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity. If a sufficiently large amount of liposomes will be modified, the solid sulfo-LC-SPDP may be added directly to the reaction mixture without preparing a stock solution in water.

3. Add 25–50 µl of the stock solution of either SPDP or LC-SPDP in DMF to each ml of the liposome suspension to be modified. If sulfo-LC-SPDP is used, add 50–100 µl of the stock solution in water to each ml of liposome suspension.
4. Mix and react for at least 30 minutes at room temperature. Longer reaction times, even overnight, will not adversely affect the modification.
5. Purify the modified liposomes from reaction by-products by dialysis or gel filtration using a column of Sephadex G-50.

The SPDP-activated liposomes may be used immediately to couple with sulfhydryl-containing molecules such as proteins (see Section 7.7, this chapter), or they may be stored in a lyophilized state in the presence of sorbitol (Friede *et al.*, 1993) for latter use.

3. Use of Glycolipids and Lectins to Effect Specific Conjugations

Glycolipids are carbohydrate-containing molecules, usually of sphingosine derivation, possessing a hydrophobic, fatty acid tail that embeds them into membrane bilayers. The hydrophilic

carbohydrate ends of these amphipathic molecules orient toward the outer aqueous phase, protruding from the bilayer surface, and thus having the capability to interact with molecules dissolved in the surrounding environment. Sphingosine glycolipids may consist of the simple glucosylcerebroside molecules (containing a single glucose residue), lactosylceramide (containing up to four glucose and galactose residues), or complex gangliosides (containing elaborate oligosaccharides that may approach the complexity of those carbohydrate "trees" found on glycoproteins). In membranes of biological origin, glycoconjugates provide sites of cellular recognition for the binding or attachment of proteins and other molecules that possess binding sites able to interact with the particular saccharides present. Such proteins, called lectins, recognize unique sugars or polysaccharide sequences within the carbohydrates of glycoconjugates.

Liposomes partially constructed of glycolipids of known carbohydrate content may be targeted by lectin molecules possessing the requisite binding properties. The liposome may be labeled in this manner with a lectin conjugate, wherein the lectin possesses another molecule covalently attached to it having secondary detection or recognition properties. For instance, a liposome containing a glycolipid may be modified by a lectin–antibody complex, producing a conjugated antibody for specific antigen-targeting applications (Figure 22.15). In addition, since lectins typically are multivalent in character, having more than one binding site for a particular carbohydrate type, they can act as multifunctional crosslinking agents to agglutinate cells or liposomes containing the proper saccharide receptors.

The advantage of this approach to liposome conjugation is that the linkage between the lectin complex and the membrane bilayer is noncovalent and reversible. The addition of a saccharide containing the proper sequence or sugar type recognized by the lectin breaks the binding

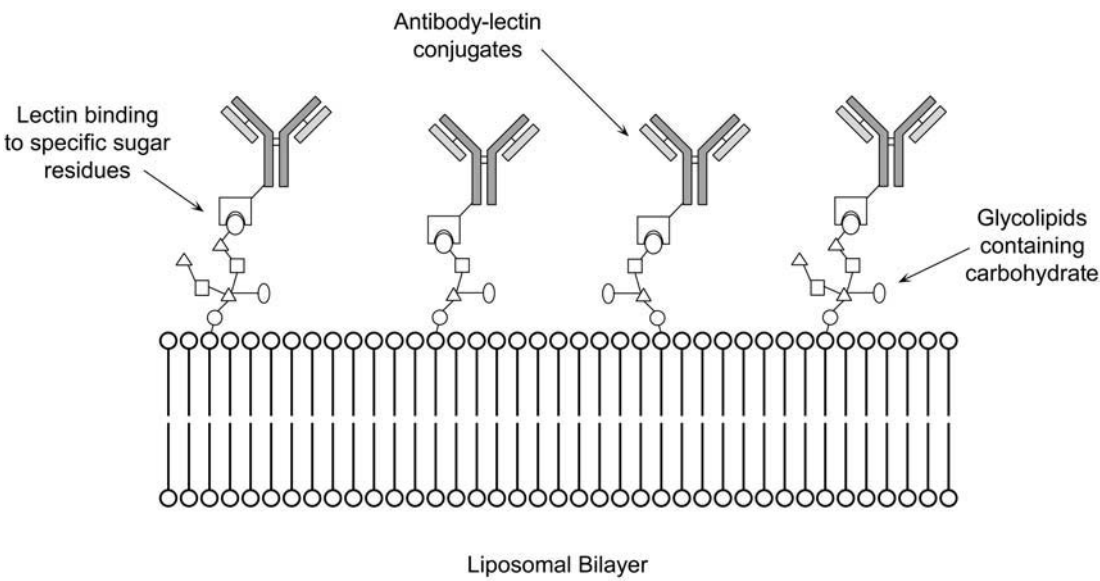


Figure 22.15 Glycolipid components included in liposome construction may be used to couple antibody molecules by using conjugates of lectins with the proper specificity for binding the sugar groups.

and releases the attached molecules. This property can be a significant disadvantage, as well, if stable linkages are desired.

Carbohydrate residues on the surface of liposomes may be used to bind selected receptor molecules on cell surfaces. This approach can provide a targeting ability for the vesicles *in vivo*, delivering drugs or toxic agents to intended cellular destinations (for review, see Leserman and Machy, 1987). Lectins also may be covalently conjugated to liposome surfaces to provide targeting capability toward cells or molecules containing the complementary carbohydrate needed for binding.

4. Antigen or Hapten Conjugation to Liposomes

Liposomes exert an adjuvant effect *in vivo*, and thus they may be used as carrier systems in the generation of a specific immune response directed against associated antigen or hapten molecules (Heath *et al*., 1976). Since the main targets of liposomes are the reticulo-endothelial system, particularly macrophages, they naturally associate with the very cells important for mediating humoral immunity. The antigenicity of a liposomal vesicle to a large degree is determined by its overall composition. Its morphology, phospholipid composition, charge, and any attached functional groups all affect the resultant antibody response (Allison and Gregoriadis, 1974; Alving, 1987; Therien and Shahum, 1989).

For instance, incorporation of beef sphingomyelin instead of egg lecithin into liposomal antigen–carrier systems can increase the antibody response to the associated antigen (Yasuda *et al*., 1977). Liposomal recipes using immune modulators such as lipid A (0.8 nmol of lipid A per μmol of phospholipid) or attaching muramyl dipeptide to the surface of the bilayer also can stimulate the immune response (Daemen *et al*., 1989). In addition, the fatty acid composition of the phospholipid components can dramatically affect liposome immunogenicity. In general, the higher the transition temperature of the associated phospholipids, the greater the immune response to the liposome. Thus, the relative order of immunogenicity related to fatty acid composition is: distearoyl > dipalmitoyl > dimyristoyl. It is also possible that the presence of a positive charge on the bilayer surface may increase the resultant immune response (Domen *et al*., 1987; Muckerheide *et al*., 1987b; Apple *et al*., 1988; Domen and Hermanson, 1992).

Two methods may be used to make immunogenic antigen–liposome or hapten–liposome complexes: (1) the molecule may be dissolved in solution and encapsulated within the vesicle construction or (2) it may be covalently coupled to the phospholipid constituents using standard crosslinking reactions (Shek and Sabiston, 1982a, b). If the antigen molecules are not chemically coupled to the liposome, then they must be entrapped within them to effect an enhancement of the antibody response. If antigen is simply mixed with pre-formed liposomal vesicles, then there is no beneficial modulation of immunogenicity (Therien and Shahum, 1989). Encapsulation of soluble or particulate vaccines into giant liposomes provides a means of extending the half-life of the vaccine molecules *in vivo* and potentiating the immune response toward the vaccine (Antimisiaris *et al*., 1993).

When haptens or antigens are covalently attached to liposomes, it is typically done through the head groups using various phospholipid derivatives and crosslinking reagents as described previously (Derksen and Scherphof, 1985). Usually, these derivatization reactions are done using PE constituents within the liposomal mixture. The primary amine on PE molecules provides an ideal functional group for activation and subsequent coupling of hapten molecules

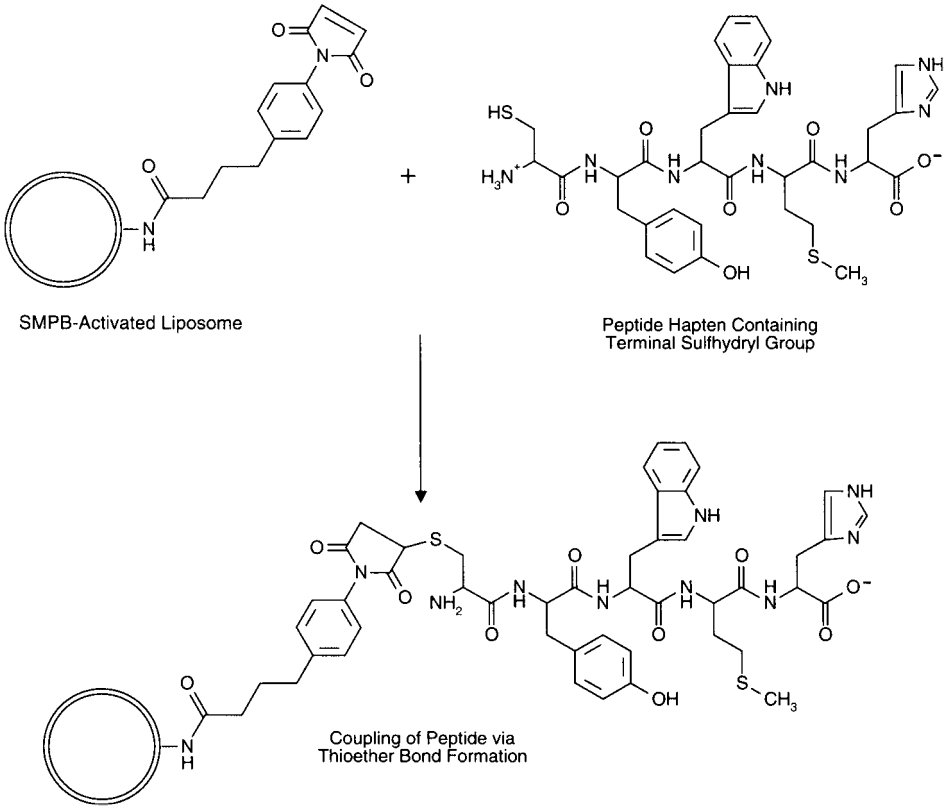


Figure 22.16 SMPB-activated liposomes may be modified with peptide hapten molecules containing cysteine thiol groups. The resultant immunogen may be used for immunization purposes to generate an antibody response against the coupled peptide.

(Shek and Heath, 1983). All of the amine-reactive activation methods discussed in this section using heterobifunctional crosslinkers may be used with PE containing liposomes to prepare immunogen conjugates. In addition, the crosslinking methods in Chapter 19, dealing specifically with hapten–carrier conjugation, should be consulted for potential use with liposomes.

The following protocols provide suggested crosslinking strategies for producing an antigen or hapten complex with liposomes. The first procedure is a simple encapsulation of antigen molecules. The second method involves the coupling of a sulfhydryl-containing peptide hapten to a liposome that had been previously activated with SMPB (Figure 22.16) (see Section 2, this chapter). They are based on the methods of Van Regenmortel *et al.* (1988).

Protocol for the Encapsulation of Antigen into Liposomal Vesicles

1. Prepare a homogeneous lipid mixture by dissolving the desired components in chloroform. A suggested recipe may be to use a mixture of DPPC:DPPG:cholesterol at a molar ratio of 7.5:2.5:5. Evaporate the chloroform using a rotary evaporator under vacuum. Maintain lipids under nitrogen or argon to prevent air oxidation.

2. Dissolve the hapten or antigen to be encapsulated at a concentration of 21 μmol/ml in degassed, nitrogen-purged 10 mM HEPES, 0.15 M NaCl, pH 6.5.
3. Create liposomal vesicles using any established method (see Section 1, this chapter) by mixing the antigen solution with the lipid mixture to obtain a final concentration of 5 mg/ml lipid in the aqueous buffer. A suggested procedure may be to redissolve the lipids in a minimum quantity of diethyl ether, and then mix the buffer with the ether phase at a ratio necessary to give a 5 mg/ml concentration of lipid in the buffer. Use sonication to emulsify the liposomal preparation. Remove diethyl ether by vacuum evaporation. Periodically mix by vortexing to maintain a homogeneous suspension of liposomes.
4. Remove free antigen from encapsulated antigen by gel filtration using a column of Sephadex G-75 or by dialysis using 10 mM HEPES, 0.15 M NaCl, pH 6.5. Store under an inert gas at 4°C in the dark until use.

Protocol for the Coupling of Peptide Haptens Containing Sulfhydryl Groups to Liposomal Vesicles

1. Prepare a homogeneous lipid mixture by dissolving in chloroform a mixture of DPPC: DPPG:cholesterol:MPB-DPPE at a mole ratio of 6.3:2.12:4.25:1.5. Preparation of MPB-activated DPPE can be found in Section 2, this chapter. Maintain all solutions under nitrogen or argon. The lipid derivative provides reactive maleimide groups for the coupling of sulfhydryl-containing molecules. Evaporate the chloroform using a rotary evaporator under vacuum.
2. Create liposomal vesicles using any established method (see Section 1, this chapter) by mixing the lipid mixture with degassed, nitrogen-purged 10 mM HEPES, 0.15 M NaCl, pH 7.0, to obtain a final concentration of 5 mg/ml lipid in the aqueous buffer. Use sonication to emulsify the liposomal preparation. Remove diethyl ether by vacuum evaporation. Periodically mix by vortexing to maintain a homogeneous suspension of liposomes.
3. Dissolve a sulfhydryl-containing peptide hapten at a concentration of 25 μmol/ml in degassed, nitrogen-purged 10 mM HEPES, 0.15 M NaCl, pH 7.0. Add the peptide solution to the liposome suspension at a molar ratio necessary to obtain at least a 5:1 excess of thiol groups to the amount of maleimide groups present (as MPB-DPPE).
4. React overnight at room temperature. Maintain an inert-gas blanket over the vessel to prevent lipid oxidation.
5. Purify the derivatized liposomes from excess peptide by gel filtration using a column of Sephadex G-75 or by dialysis. Store the immunogenic vesicles at 4°C under nitrogen or argon and protected from light until use.

5. Preparation of Antibody–Liposome Conjugates

Covalent attachment of antibody molecules to liposomes can provide a targeting capacity to the vesicle that can modulate its binding to specific antigenic determinants on cells or to molecules in solution. Antibody-bearing liposomes may possess encapsulated components that can be used for detection or therapy (Figure 22.17). For instance, fluorescent molecules encapsulated within antibody-targeted vesicles can be used as imaging tools or in flow cytometry

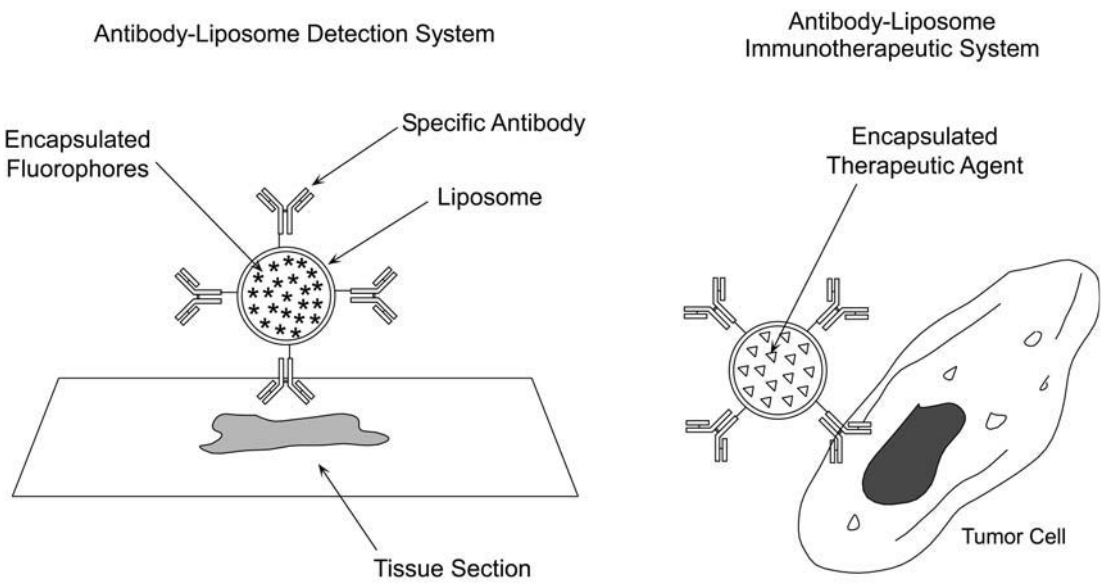


Figure 22.17 Antibody–liposome conjugates may be used as targeting reagents for detection or therapeutic applications. The liposome may be constructed to contain fluorescent molecules for detection purposes or bioactive agents for therapy. The antibody component targets the complex for binding to specific antigenic determinants.

(Truneh *et al*., 1987). Specific antibodies coupled to the vesicle surface can improve diagnostic assays involving agglutination of latex particles (Kung *et al*., 1985). Liposomes possessing antibodies directed against tumor cell antigens can deliver encapsulated toxins or drugs to the associated cancer cells, effecting toxicity and cell death (Heath *et al*., 1983; Heath *et al*., 1984; Matthay *et al*., 1984; Straubinger *et al*., 1988).

Encapsulation of chemotherapeutic agents within lipid bilayers reduces systemic toxicity and local irritation often caused by anticancer drugs (Gabizon *et al*., 1986). The liposomal membrane acts as a slow-release agent so that cytotoxic components do not come into contact with non-tumor cells. Liposome binding to cells causes internalization and release of the encapsulated drugs. Antibody targeting can increase the likelihood of vesicle binding to the desired tumor cells.

However, there are problems associated with the use of antibody–liposome conjugates for drug delivery *in vivo*. Particularly, since lipid vesicles are huge compared to similar immunotoxin conjugates (Chapter 21), their passage to particular tissue destinations may be difficult or impossible. Liposomes are almost entirely limited to the reticuloendothelial system. Their ability to pass through tissue barriers to target cells in other parts of the body is limited by their size. If liposomal conjugates can reach their intended destination, their contents are delivered to the cells by endocytosis. Endocytic vesicles arising from the surface of cells have diameters in the range of 1,000–1,500 Å. This limits the size of liposomes that can be used to small vesicles that can bind to the surface of a cell and be internalized efficiently. Large liposomes, by contrast, will not be internalized and therefore not be able to deliver their contents (Leserman and Machy, 1987).

The methods for coupling antibody molecules to liposomal surfaces are not unlike those described for general protein coupling (Section 7, this chapter). Antibodies may be coupled through sulfhydryl residues using liposomes-containing PE groups that are derivatized with heterobifunctional crosslinkers such as SMCC (Chapter 5, Section 1.3), MBS (Chapter 5, Section 1.4), SMPB (Chapter 5, Section 1.6), SIAB (Chapter 5, Section 1.5), SPDP (Chapter 5, Section 1.1), and heterobifunctional PEG-based crosslinkers (Chapter 18, Section 2). They also may be coupled through their amine groups using reductive amination to periodate-oxidized glycolipids (Sections 2 and 7.6, this chapter).

6. Preparation of Biotinylated or Avidin-Conjugated Liposomes

Liposome conjugates may be used in various immunoassay procedures. The lipid vesicle can provide a multivalent surface to accommodate numerous antigen–antibody interactions and thus increase the sensitivity of an assay. At the same time, it can function as a vessel to carry encapsulated detection components needed for the assay system. This type of enzyme-linked immunosorbent assay (ELISA) is called a liposome immunosorbent assay or LISA. One method of using liposomes in an immunoassay is to modify the surface so that it can interact to form biotin–avidin or biotin–streptavidin complexes. The avidin–biotin interaction can be used to increase detectability or sensitivity in immunoassay tests (Chapter 23) (Savage *et al*., 1992).

Liposomes containing biotinylated phospholipid components can be used in a bridging assay system with avidin and a biotinylated antibody molecule, creating large multivalent complexes able to bind antigen (Plant *et al*., 1989) (Figure 22.18). The inside of the vesicles may contain fluorescent detection reagents that can be used to localize or quantify target analytes. One small liposome provides up to 10^5 molecules of fluorophore to allow excellent detectability of a binding event. LISA systems using biotinylated liposomes to detect antigen molecules can increase the sensitivity of an immunoassay up to 100-fold over that obtainable using traditional antibody–enzyme ELISAs.

Biotinylated liposomes usually are created by modification of PE components with an amine-reactive biotin derivative, for example NHS-LC-Biotin (Chapter 11, Section 1). The NHS ester reacts with the primary amine of PE residues, forming an amide bond linkage (Figure 22.19). A better choice of biotinylation agent may be to use the NHS-PEG$_n$-biotin compounds (Chapter 18), because the hydrophilic PEG spacer provides better accessibility in the aqueous environment than a hydrophobic biotin spacer. Since the modification occurs at the hydrophilic end of the phospholipid molecule, after vesicle formation the biotin component protrudes out from the liposomal surface. In this configuration, the surface-immobilized biotins are able to bind (strept)avidin molecules present in the outer aqueous medium.

However, since many of the traditional biotinylation reagents, such as NHS-LC-biotin contain hydrophobic spacers, their use with amphipathic liposomal constructions may not be entirely appropriate. A better choice may be to use a hydrophilic PEG-based biotin compound that creates a water-soluble biotin modification on the outer aqueous surface of the liposome bilayer. Biotinylation reagents of this type are discussed in Chapter 18, Section 3.

Biotinylation may be done before or after liposome formation, but having a stock supply of biotin-modified PE is an advantage, since it can then be used to test a number of liposomal recipes. In addition, only a very small percent of the total lipid should be biotinylated to prevent avidin-induced aggregation in the absence of antigen. It is difficult to control precisely

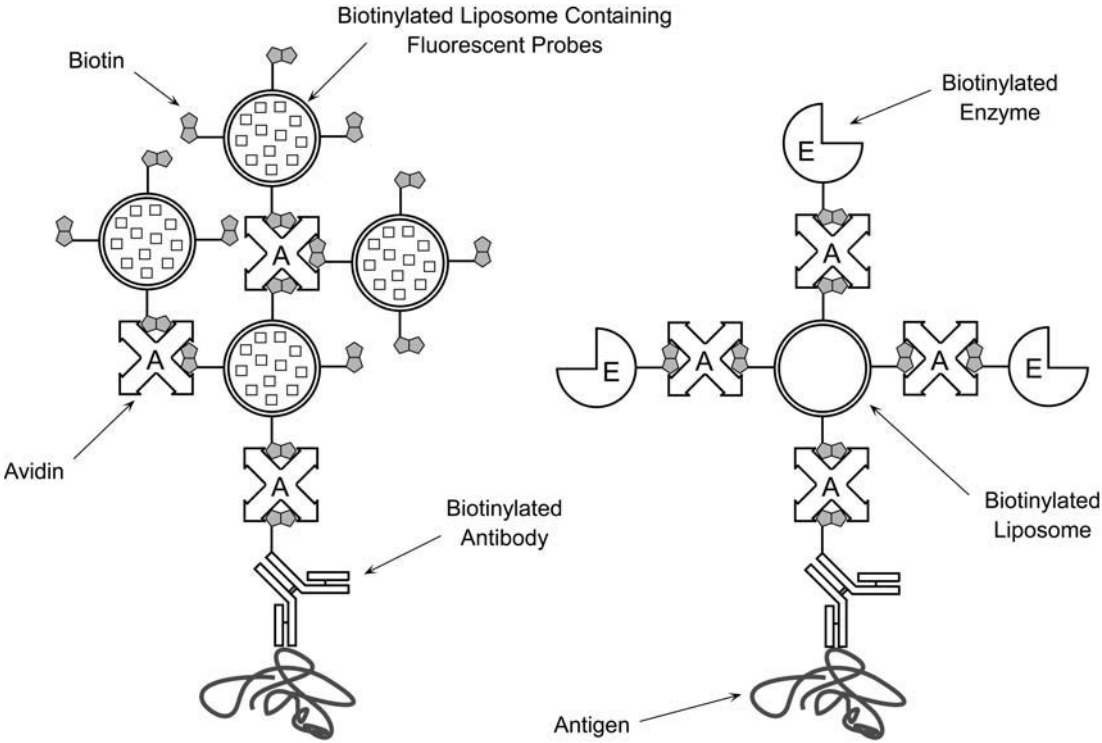


Figure 22.18 Biotinylated liposomes may be used in immunoassay systems to enhance the signal for detection or measurement of specific analytes. The liposome components may be constructed to include fluorescent molecules to facilitate detection of antigens within tissue sections.

the biotin content if direct biotinylation of intact liposomes is done. Using pure biotinylated phospholipid allows incorporation of discrete amounts of biotin binding sites into the final liposomal membrane. The preparation of biotinylated PE (B-PE) can be done similar to the methods described for activation of PE with SMPB (Section 2, this chapter) or it may be obtained commercially.

The following method for the formation of a biotinylated liposome is adapted from Plant *et al*., 1989). It assumes prior production of B-PE.

Protocol

1. Prepare a biotinylated liposome construct by first dissolving in chloroform, the lipids DMPC:cholesterol:dicetylphosphate (Sigma) at mole ratios of 5:4:1, and adding to this solution 0.1 mol percent B-PE. Larger mole ratios of B-PE to total lipid may result in nonspecific aggregation of liposomes in the presence of avidin. Maintain all lipids under an inert atmosphere to prevent oxidation.
2. Evaporate 2 μmol of total homogenized lipid in chloroform using a stream of nitrogen or a rotary vacuum evaporator.

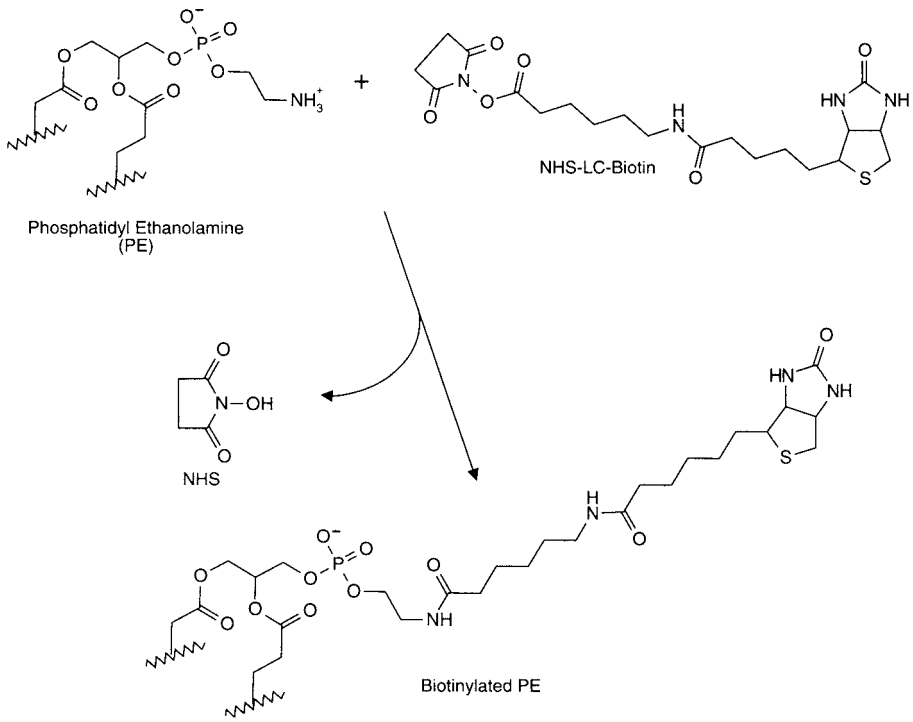


Figure 22.19 Biotinylated liposomes may be formed using biotinylated PE. Reaction of NHS-LC-biotin with PE results in amide bond linkages and a long spacer arm terminating in a biotin group.

3. Redissolve the dried lipid in 50 µl of dry isopropanol.
4. Take the lipid solution up into a syringe and inject it into 1 ml of degassed, nitrogen-purged 20 mM Tris, 0.15 M NaCl, pH 7.4, which is being vigorously stirred using a vortex mixer. To encapsulate a fluorescent dye using this procedure, include 100 mM 5,6-carboxyfluorescein (or another suitable fluorophore) in the buffer solution before adding the lipids. The fluorescent probe used in the encapsulation procedure should be chemically nonreactive so that no lipid components are covalently modified during the process.

The biotinylated liposomes prepared by this procedure may be stored under an inert-gas atmosphere at 4°C for long periods without degradation.

7. Conjugation of Proteins to Liposomes

Covalent attachment of proteins to the surface of liposomal bilayers is done through reactive sites created on the head groups of phospholipids with the intermediary use of a crosslinker or other activating agent. The lipid functional groups described in Section 1 of this chapter are modified according to the methods discussed in Section 2 to be reactive toward specific target

groups in proteins. Conjugation of liposomes with proteins may be done with homobifunctional or heterobifunctional crosslinking reagents, carbodiimides, reductive amination, by NHS ester activation of carboxylates, or through the noncovalent use of the avidin–biotin interaction.

Characterizing the resultant complex for the amount of protein per liposome is somewhat more difficult than in other protein conjugation applications. The protein–liposome composition is highly dependent on the size of each liposomal particle, the amount of protein charged to the reaction, and the mole quantity of reactive lipid present in the bilayer construction. An approach to solving this problem is presented by Hutchinson *et al.* (1989). In analyzing at least 17 different protein–liposome preparations, the ratio of protein:lipid content (μg protein/μg lipid) in most of the complexes ranged from a low of about 4 to as much as 675. In some instances, however, up to 6,000 molecules of a particular protein could be incorporated into each liposome.

Coupling of protein molecules to liposomes occasionally may induce vesicle aggregation. This may be due to the unique properties or concentration of the protein used, or it may be a result of liposome-to-liposome crosslinking during the conjugation process. Adjusting the amount of protein charged to the reaction as well as the relative amounts of crosslinking reagents employed may have to be done to solve an aggregation problem.

The following sections present suggested protocols for creating protein-bearing liposomes. Each method utilizes specific lipid modifications to form reactive groups capable of targeting amines, sulfhydryls, aldehydes, or carboxylates on the protein molecules.

7.1. Coupling via the NHS Ester of Palmitic Acid

Huang *et al.* (1980) coupled monoclonal antibodies to liposomes using an NHS ester modification of palmitic acid incorporated into the bilayer construction (Lapidot *et al.*, 1967). The NHS ester reacts with amine groups on the protein molecule, producing stable amide bond linkages (Figure 22.20). The specificity of the antibody-bearing liposomes for mouse L-929 cells was documented, illustrating the preservation of antibody binding activity.

Protocol

Preparation of NHS–Palmitate

1. Dissolve 3.45 mg of N-hydroxysuccinimide (NHS) in 30 ml dry ethyl acetate.
2. Add 30 mmol of palmitic acid to the NHS solution. Maintain a nitrogen blanket over the solution.
3. Dissolve 6.18 g of dicyclohexyl carbodiimide (DCC; Chapter 3, Section 1.4) in 10 ml of ethyl acetate and add it to the NHS/palmitic acid solution.
4. React overnight at room temperature under a nitrogen blanket.
5. Remove the insoluble dicyclohexyl urea (DCU) by-product by filtration using a glass fiber filter pad and vacuum.
6. Remove solvent from the filtered solution by using a rotary evaporator under vacuum.
7. The NHS–palmitate may be purified by recrystallization using ethanol. Dissolve the activated fatty acid in a minimum quantity of hot ethanol. Immediately upon dissolving, filter it through a filter funnel containing a fluted glass fiber filter pad, both of which have

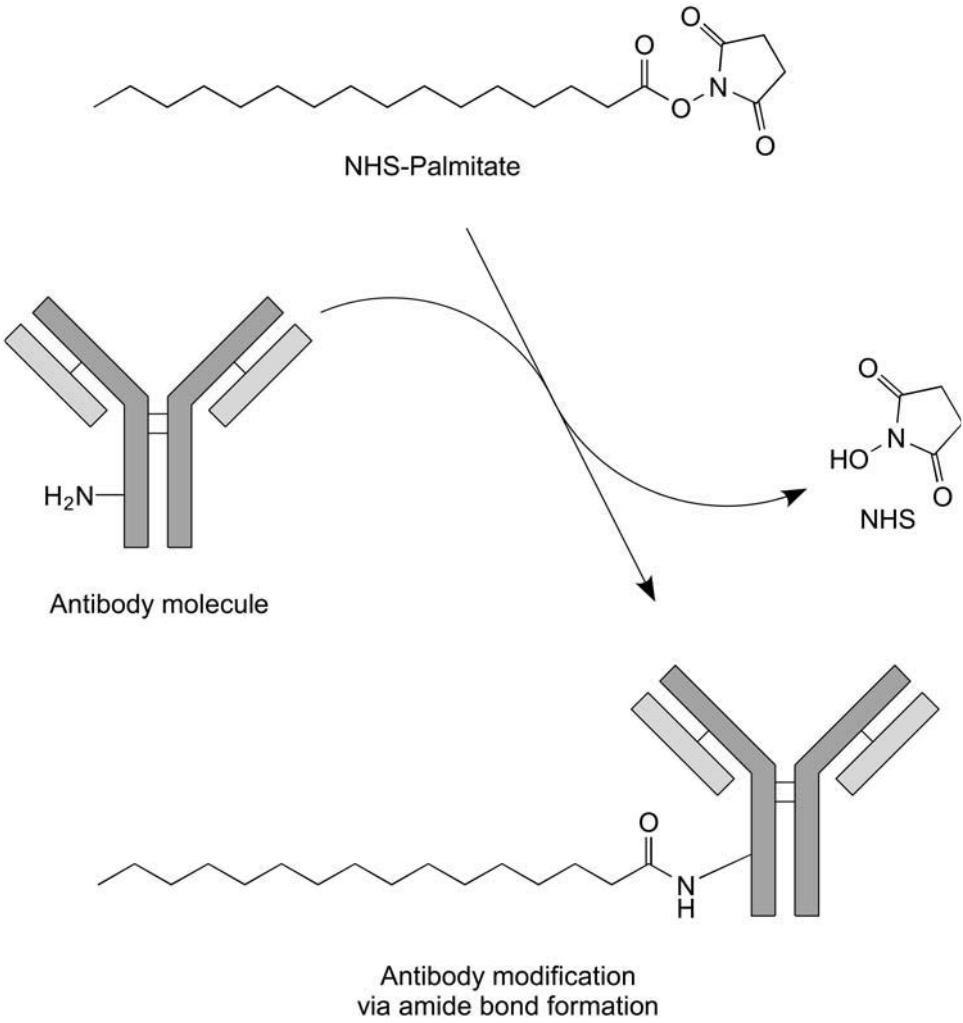


Figure 22.20 The NHS ester derivative of palmitic acid may be used to couple antibody molecules through amide bonds. These complexes then may be incorporated into liposomes.

been warmed to the same temperature as the ethanol solution. Allow the NHS–palmitate to recrystallize overnight at room temperature. Remove solvent from the recrystallized solid by filtration. Dry under vacuum in a desiccator.

8. Analyze the NHS–palmitate for purity using TLC on silica plates. Develop using a solvent mixture of chloroform:petroleum diethyl ether (bp 40–60°C) of 8:2. Excess NHS and NHS–palmitate may be detected by staining with 10 percent hydroxylamine in 0.1 N NaOH, followed after 2 minutes by a 5 percent solution of $FeCl_3$ in 1.2 N HCl (creates red colored spots).

Coupling of Protein to NHS–Palmitate

1. Add 2 mg of protein to 44 μg NHS–palmitate in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4, containing 2 percent deoxycholate.
2. Incubate at 37°C for 10 hours.
3. Remove excess palmitic acid by chromatography on a column of Sephadex G-75. Use PBS, pH 7.4, containing 0.15 percent deoxycholate to perform the gel filtration. Collect the fractions containing derivatized protein, as monitored by absorbance at 280 nm.

Addition of Protein–Palmitate Conjugate to Liposomal Membranes

Since the protein–palmitate derivative can't be dissolved in organic solvent during homogenization of lipid to form liposomal membranes, it must be inserted into intact liposomes by detergent dialysis.

1. Construct a liposome by dissolving the desired lipids in chloroform to homogenize fully the mixture, drying them to remove solvent, and using any established method of forming bilayer vesicles in aqueous solution (i.e., sonication; see Section 1, this chapter).
2. Add protein–palmitate conjugate to the formed liposomes in a ratio of 20:1 (w/w). Add concentrated deoxycholate to give a final concentration of 0.7 percent. Mix thoroughly using a vortex mixer.
3. Dialyze the liposome preparation against PBS, pH 7.4.
4. The liposome vesicles may be characterized for size by chromatography on a column of Sepharose 4B.

7.2. Coupling via Biotinylated PE Lipid Derivatives

Biotinylated PE (B-PE) incorporated into liposomal membranes can be used to interact noncovalently with (strept)avidin–protein conjugates or with other biotinylated proteins using (strept)avidin as a bridging molecule (Plant *et al.*, 1989). It is important that a long-chain spacer be used in constructing the B-PE derivative to allow enough spatial separation from the bilayer surface to accommodate avidin docking (Hashimoto *et al.*, 1986). Thus, any biotinylated protein can be coupled to the liposome surface through the strength of the (strept)avidin–biotin interaction. Section 6 (this chapter) describes the preparation of B-PE derivatives and their addition into vesicle construction. Incubation of (strept)avidin conjugates or (strept)avidin plus a biotinylated protein with the biotinylated liposome in PBS, pH 7.4, will form essentially nonreversible complexes, immobilizing the proteins to the outer surface of the bilayers. This method can be used to couple biotin-modified antibody molecules to liposomes (Figure 22.21). Removal of noncomplexed protein may be done using gel filtration chromatography on a column of Sephadex G-50 or G-75.

7.3. Conjugation via Carbodiimide Coupling to PE Lipid Derivatives

Underivatized PE in liposomal membranes contains an amine group that can participate in the carbodiimide reaction with carboxylate groups on proteins or other molecules (Dunnick *et al.*,

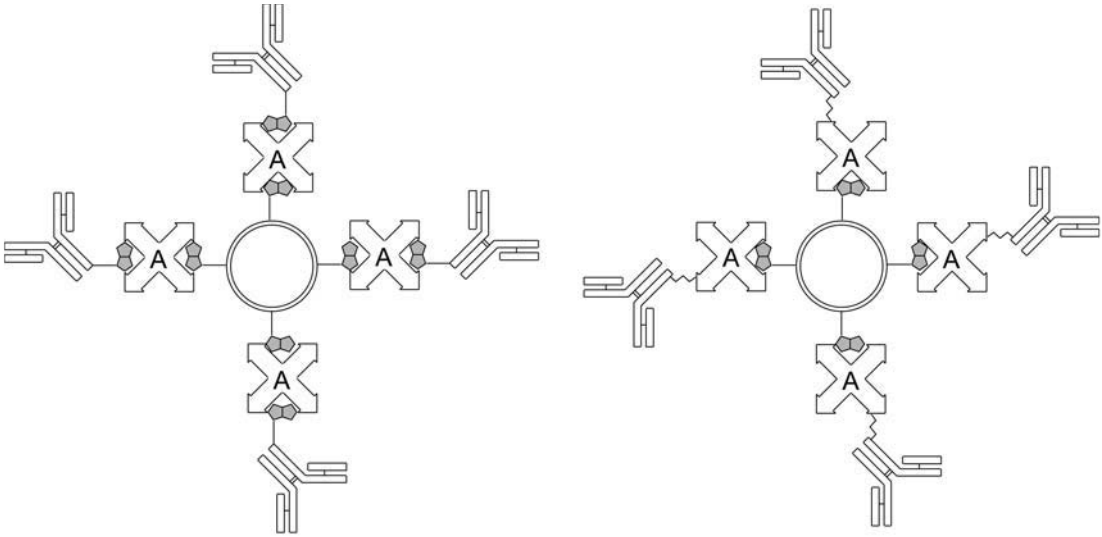


Figure 22.21 Antibodies may be conjugated to liposomes using an indirect approach incorporating a (strept)avidin–biotin system. Biotinylated liposomes may be complexed with biotinylated antibodies using (strept)avidin as a bridging molecule or may be complexed with an antibody–(strept)avidin conjugate.

1975). The water-soluble carbodiimide EDC (Chapter 3, Section 1.1) activates carboxylate groups to form active-ester intermediates that can react with PE to form an amide linkage. Unfortunately, EDC coupling of proteins to surfaces often results in considerable protein-to-protein crosslinking, since proteins contain an abundance of both amines and carboxylates. There also is potential for vesicle aggregation by proteins coupling to more than one liposome. Martin *et al.* (1990) suggest avoiding this polymerization problem by first blocking the amine groups of the protein with citraconic acid, which has been used successfully with antibodies (Jansons and Mallett, 1980).

However, even with the potential for protein–protein conjugation, carbodiimide coupling of peptides and proteins to liposomes can be done with EDC without blocking polypeptide amines. The approach is similar to that described for the EDC conjugation of hapten molecules to carrier proteins to form immunogen complexes (Chapter 19, Section 3). Thus, this method may be used to prepare peptide hapten–liposome conjugates for immunization purposes (Figure 22.22). The procedure also works particularly well for coupling molecules containing only carboxylates to the amines on the liposomes.

Protocol

1. Prepare liposomes containing PE by any desired method. For instance, the common recipe mentioned in Section 1 (this chapter) that involves mixing PC:cholesterol:PG:PE in a molar ratio of 8:10:1:1 may be used. Thoroughly emulsify the liposome construction to obtain a good population of SUVs. The final liposome suspension should be in 20 mM sodium phosphate, 0.15 M NaCl, pH 7.2. Adjust the concentration to about 5 mg lipid/ml buffer.

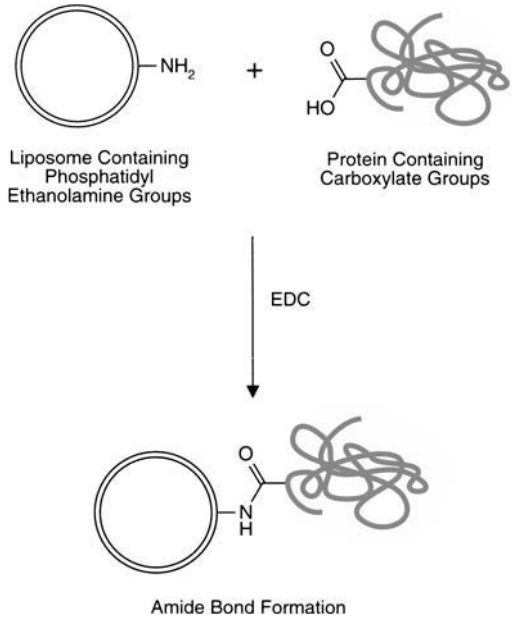


Figure 22.22 A protein may be conjugated with a liposome-containing PE groups using a carbodiimide reaction with EDC.

2. Add the protein or peptide to be conjugated to the liposome suspension. The protein may be dissolved first in PBS, pH 7.2, and an aliquot added to the reaction lipid mixture. The amount of protein to be added can vary considerably, depending on the abundance of the protein and the desired final density required. Reacting from 1 mg protein per ml liposome suspension up to about 20 mg protein/ml can be done.
3. Add 10 mg EDC per ml of lipid/protein mixture. Solubilize the carbodiimide using a vortex mixer.
4. React for 2 hours at room temperature. If liposome aggregation or protein precipitation occurs during the crosslinking process, scale back the amount of EDC added to the reaction.
5. Purify the conjugate by gel filtration using a column of Sephadex G-75.

7.4. Conjugation via Glutaraldehyde Coupling to PE Lipid Derivatives

Glutaraldehyde is among the earliest homobifunctional crosslinkers employed for protein conjugation (Chapter 4, Section 6.2). It reacts with amine groups through several routes, including the formation of Schiff base linkages which can be reduced with borohydride or cyanoborohydride to create stable secondary amine bonds. Although very efficient in reacting with proteins, glutaraldehyde typically causes extensive polymerization accompanied by precipitation of high-molecular-weight oligomers. Even with this significant disadvantage, the reagent is still used routinely in protein conjugation techniques.

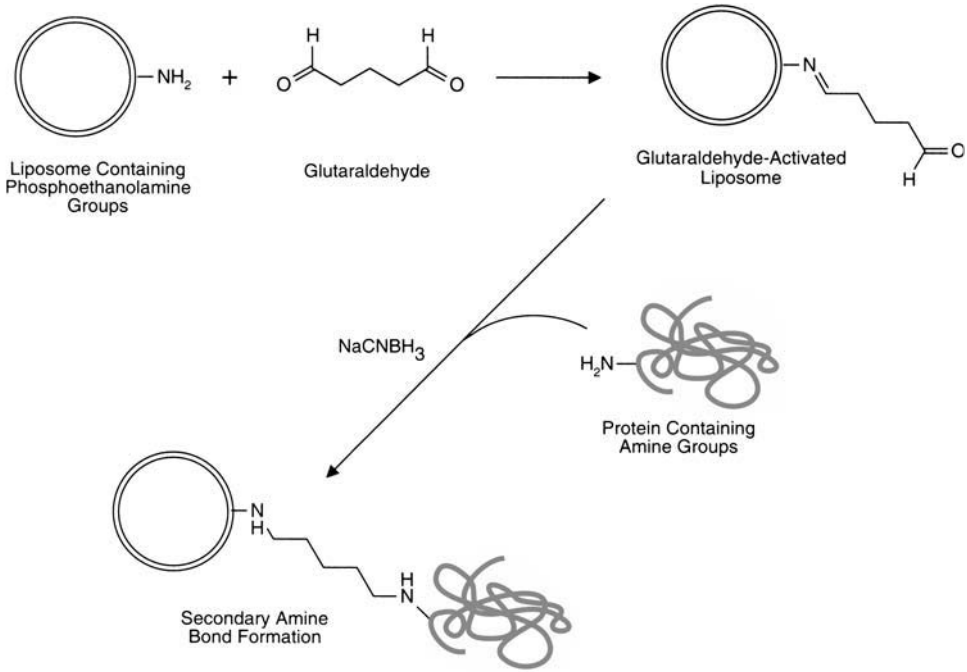


Figure 22.23 Glutaraldehyde activation of PE-containing liposomes may be used to couple protein molecules.

Liposomes containing PE residues can be reacted with glutaraldehyde to form an activated surface possessing reactive aldehyde groups. A 2-step glutaraldehyde reaction strategy is probably best when working with liposomes, since precipitated protein would be difficult to remove from a vesicle suspension.

The following protocol describes the 2-step method wherein the liposome is glutaraldehyde-activated, purified away from excess crosslinker, and then coupled to a protein by reductive amination (Figure 22.23).

Protocol

1. Prepare a liposome suspension, containing PE, at a total-lipid concentration of 5 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8. Maintain all lipid-containing solutions under an inert gas atmosphere. Degas all buffers and bubble them with nitrogen or argon prior to use.
2. Add glutaraldehyde to this suspension to obtain a final concentration of 1.25 percent.
3. React overnight at room temperature under a nitrogen blanket.
4. Purify the activated liposomes from excess glutaraldehyde by gel filtration (using Sephadex G-50) or by dialysis against PBS, pH 6.8.
5. Dissolve the protein or peptide to be conjugated at a concentration of 10 mg/ml in 0.5 M sodium carbonate, pH 9.5. Mix the activated liposome suspension with the polypeptide solution at the desired molar ratio to effect the conjugation. Mixing the equivalent of 4 mg of protein per mg of total lipid usually results in acceptable conjugates.

6. React overnight at 4°C under an atmosphere of nitrogen.
7. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.

7.5. Conjugation via DMS Crosslinking to PE Lipid Derivatives

Dimethyl suberimidate (DMS) is a homobifunctional crosslinking agent containing amine-reactive imidoester groups on both ends. The compound is reactive toward the ε-amine groups of lysine residues and N-terminal α-amines in the pH range of 7–10 (pH 8–9 is optimal). The resulting amidine linkages are positively charged at physiological pH, thus maintaining the positive charge contribution of the original amine.

Bis-imidoesters like DMS may be used to couple proteins to PE-containing liposomes by crosslinking with the amines on both molecules (Figure 22.24). However, single-step crosslinking procedures using homobifunctional reagents are particularly subject to uncontrollable polymerization of protein in solution. Polymerization is possible because the procedure is done with the liposomes, protein, and crosslinker all in solution at the same time.

The reaction is carried out in 0.2 M triethanolamine, pH 8.2. DMS should be the limiting reagent in the reaction to avoid blocking all amines on both molecules with only one end of the

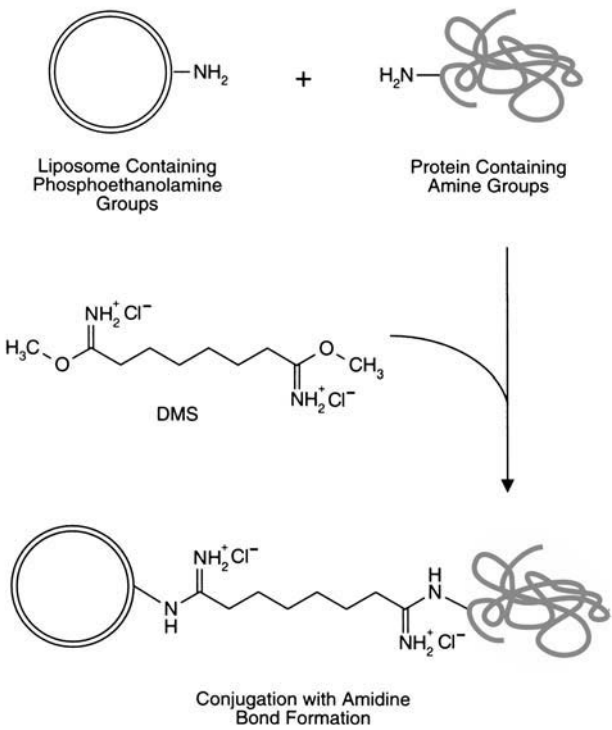


Figure 22.24 The homobifunctional crosslinker DMS may be used to conjugate PE-containing liposomes with proteins via amidine bond formation.

crosslinker, thus eliminating any conjugation. The amounts of total lipid and protein in solution may have to be adjusted to optimize each conjugation reaction and avoid precipitation of protein or aggregation of liposomes.

7.6. Conjugation via Periodate Oxidation Followed by Reductive Amination

Periodate-oxidized liposomes which contain glycolipid moieties may be used to couple proteins and other amine-containing molecules by reductive amination. Section 2 (this chapter) describes the oxidative procedure that results in the formation of reactive aldehyde groups on the liposomal surface. Amine-containing polypeptides form Schiff base linkages with the aldehyde groups under alkaline conditions. The addition of a reducing agent such as borohydride or cyanoborohydride reduces the labile Schiff bases to form stable secondary amine bonds (Figure 22.25).

The following generalized method is based on the procedure described by Heath *et al.* (1981) for the coupling of immunoglobulins to liposomes containing glycosphingolipids.

Protocol

1. Periodate-oxidize a liposome suspension containing glycolipid components according to Section 2 (this chapter). Adjust the concentration of total lipid to about 5 mg/ml.
2. Dissolve the protein to be coupled in 20 mM sodium borate, 0.15 M NaCl, pH 8.4, at a concentration of at least 10 mg/ml.
3. Add 0.5 ml of protein solution to each ml of liposome suspension with stirring.
4. Incubate for 2 hours at room temperature to form Schiff base interactions between the aldehydes on the vesicles and the amines on the protein molecules.

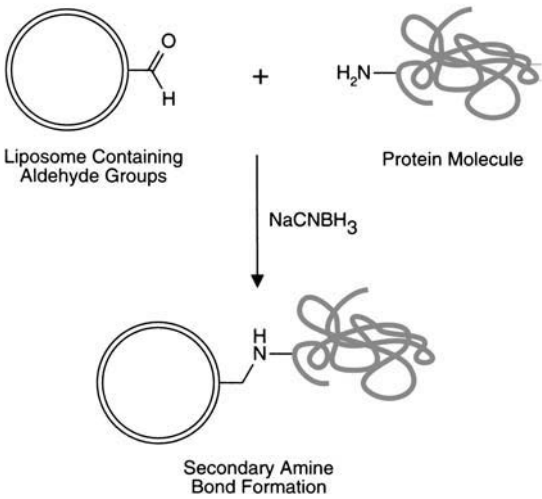


Figure 22.25 Glycolipids incorporated into liposomes may be oxidized with periodate to produce aldehydes suitable for coupling proteins via reductive amination.

5. In a fume hood, dissolve 125 mg of sodium cyanoborohydride in 1 ml water (makes a 2 M solution). *Caution: Highly toxic compound; handle with care*. This solution may be allowed to sit for 30 minutes to eliminate most of the hydrogen-bubble evolution that could affect the vesicle suspension.
6. Add 10 µl of the cyanoborohydride solution to each ml of the liposome reaction.
7. React overnight at 4°C.
8. Remove unconjugated protein and excess cyanoborohydride by gel filtration using a column of Sephadex G-50 or G-75.

7.7. Conjugation via SPDP-Modified PE Lipid Derivatives

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) is one of the most popular heterobifunctional crosslinking agents available, especially for protein conjugation (Chapter 5, Section 1.1). The activated NHS ester end of SPDP reacts with amine groups in proteins and other molecules to form an amide linkage (Figure 22.26). The 2-pyridyldithiol group at the other end reacts with sulfhydryl residues to form a disulfide linkage with sulfhydryl-containing molecules (Carlsson *et al*., 1978).

SPDP also is a popular choice for coupling sulfhydryl-containing molecules to liposomes. PE residues in vesicles may be activated with this crosslinker to form pyridyl disulfide derivatives that can react with sulfhydryls to form disulfide linkages. Unlike the iodoacetyl- and maleimide-based crosslinkers discussed previously, the linkage formed with SPDP is reversible by simple disulfide reduction. Pure PE may be activated with SPDP prior to its incorporation into a liposome,

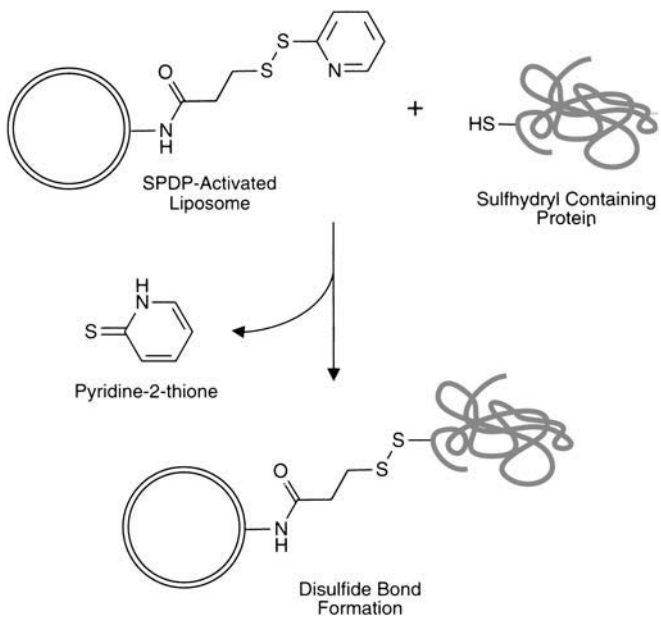


Figure 22.26 SPDP-activated liposomes can be used to couple sulfhydryl-containing proteins, forming disulfide linkages.

or intact liposomes containing PE may be activated using the methods described in Section 2 (this chapter). Activation of PE with an SPDP crosslinker forms the intermediate reactive pyridyldithiopropionate–PE (PDP–PE) derivative. Stearylamine also can be activated with SPDP to be used in liposome conjugation (Goundalkar *et al.*, 1983). If the long-chain version, sulfo-LC-SPDP, is used with intact vesicles, the crosslinker will be water-soluble and may be added directly to the buffered suspension without prior organic solvent dissolution. The negatively charged sulfonate group on its NHS ring prevents the reagent from penetrating the hydrophobic region of the lipid bilayer. Thus, only the outer surface of the liposomes will be modified. Using preactivated PE, both inner and outer surfaces end up containing reactive pyridyl disulfide groups. If liposome-sequestered components have the potential to react with this functional group or are sensitive, activation of intact liposomes with sulfo-LC-SPDP may be the better tactic.

The following protocol is a suggested method for coupling sulfhydryl-containing proteins to SPDP-activated vesicles.

1. Prepare a 5 mg/ml liposome suspension containing a mixture of PC:cholesterol:PG:PDP-PE in molar ratios of 8:10:1:1. The emulsification may be done by any established method (Section 1, this chapter). Suspend the vesicles in 50 mM sodium phosphate, 0.15 M NaCl, 10 mM ethylenediamine triacetic acid EDTA, pH 7.2.
2. Add at least 5 mg/ml of a sulfhydryl-containing protein or other molecule to the SPDP-modified vesicles to effect the conjugation reaction. Molecules lacking available sulfhydryl groups may be modified to contain them by a number of methods (Chapter 1, Section 4.1). The conjugation reaction should be done in the presence of at least 10 mM EDTA to prevent metal-catalyzed sulfhydryl oxidation.
3. React overnight with stirring at room temperature. Maintain the suspension in a nitrogen or argon atmosphere to prevent lipid oxidation.
4. The modified liposomes may be separated from excess protein by gel filtration using Sephadex G-75 or by centrifugal floatation in a polymer gradient (Derksen and Scherphof, 1985).

7.8. Conjugation via SMPB-Modified PE Lipid Derivatives

Succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB, Chapter 5, Section 1.6), is a heterobifunctional crosslinking agent that has an amine-reactive NHS ester on one end and a sulfhydryl-reactive maleimide group on the other. Conjugates formed using SMPB are linked by stable amide and thioether bonds.

SMPB can be used to activate PE residues to contain sulfhydryl-reactive maleimide groups (Section 2, this chapter). Lipid vesicles formed with reactive maleimidophenylbutyrate–PE (MPB–PE) components thus can couple proteins through available —SH groups, forming thioether linkages (Derksen and Scherphof, 1985) (Figure 22.27). A comparison with SPDP-produced conjugates concluded that SMPB formed more stable complexes that survived in serum for longer periods (Martin and Papahadjopoulos, 1982). The following protocol is a generalized method for the conjugation of proteins to SMPB-activated liposomes.

1. Prepare a liposome suspension, containing MPB–PE, at a total lipid concentration of 5 mg/ml in 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2. Activation of DPPE with SMPB is

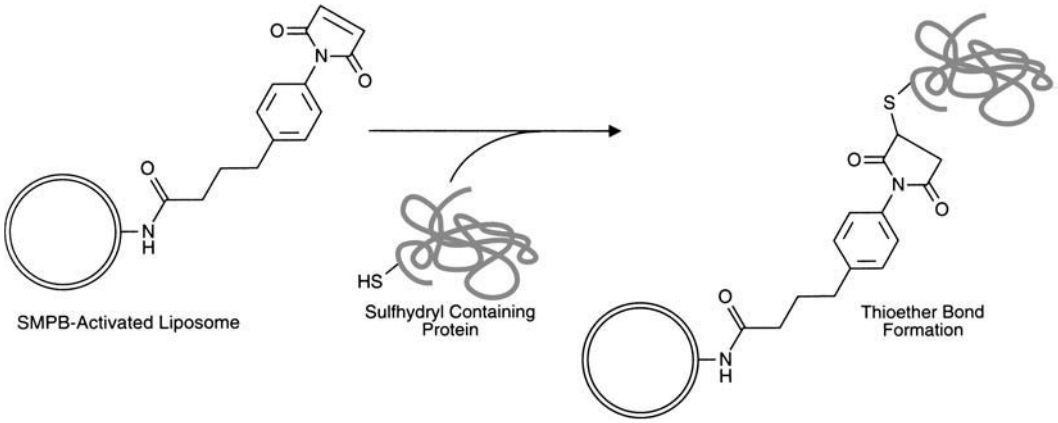


Figure 22.27 SMPB-activated liposomes may be used to couple thiol-containing protein molecules, forming stable thioether linkages.

described in Section 2, this chapter. A suggested lipid composition for vesicle formation is PC:cholesterol:PG:MPB–PE mixed at a molar ratio of 8:10:1:1. The presence of relatively high levels of cholesterol in the liposomal recipe dramatically enhances the conjugation efficiency of the component MPB–PE groups (Martin *et al.*, 1990). Any method of emulsification to create liposomes of the desired size and morphology may be used (Section 1, this chapter).

2. Dissolve a sulfhydryl-containing protein at a concentration of at least 5 mg/ml in 0.05 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. The sulfhydryl groups on the protein molecule may be indigenous or created by any of the methods described in Chapter 1, Section 4.1.
3. Mix the protein solution with the liposome suspension in equal volume amounts.
4. React overnight at room temperature with stirring. Maintain an atmosphere of nitrogen over the reaction to prevent lipid oxidation.
5. Separate unreacted protein from modified liposomes by gel filtration using a column of Sephadex G-75 or by centrifugal floatation in a polymer gradient (Derksen and Scherphof, 1985).

7.9. Conjugation via SMCC-Modified PE Lipid Derivatives

Succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is a heterobifunctional crosslinker with significant utility in crosslinking proteins, particularly in the preparation of antibody–enzyme (Chapter 20) and hapten–carrier (Chapter 19) conjugates (Hashida and Ishikawa, 1985; Dewey *et al.*, 1987). It is normally used in a two-step crosslinking procedure, wherein the NHS ester end of the reagent first is reacted with primary amine groups on proteins or other molecules to form stable amide bonds. This creates a reactive intermediate containing terminal maleimide groups on the modified molecule. The maleimide end is specific for coupling to sulfhydryls when the reaction pH is in the range of 6.5–7.5 (Smyth *et al.*, 1964). Addition of a sulfhydryl-containing protein forms a stable thioether linkage with the SMCC-activated molecule.

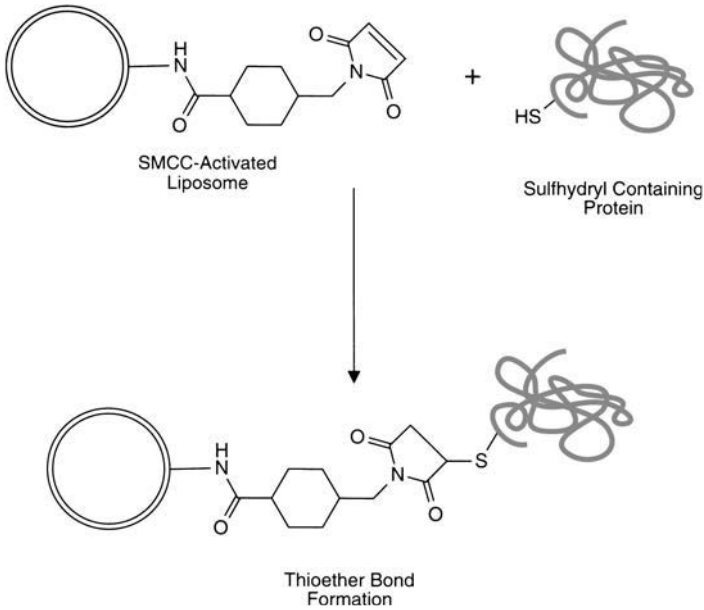


Figure 22.28 The reaction of an SMCC-activated liposome with a sulfhydryl-containing protein forms stable thioether bonds.

In a similar manner, PE may be activated through its head-group primary amine to possess reactive maleimide groups capable of coupling sulfhydryl-containing proteins to liposomes (Figure 22.28). The method of derivatizing DPPE with SMCC is essentially the same as that described for SMPB (Section 2, this chapter). SMCC, however, contains a more stable maleimide-reactive group toward hydrolysis in aqueous reaction environments, due to the proximity of an aliphatic cyclohexane ring rather than the aromatic phenyl group of SMPB. In protein conjugation to liposomes, this stability may translate into higher activity and more efficient crosslinking. A general protocol for the coupling of sulfhydryl-containing proteins to liposomes containing SMCC–PE is essentially the same as that described previously for SMPB (Section 7.8, this chapter).

7.10. Conjugation via Iodoacetate-Modified PE Lipid Derivatives

Iodoacetate derivatives have been used for decades to block or crosslink sulfhydryl groups in proteins and other molecules (Chapter 1, Section 5.2). At mildly alkaline pH values (pH 8–8.5), iodoacetyl derivatives are almost entirely selective toward the cysteine —SH groups in proteins. Disulfide reduction or thiolation reagents can be used to create the required sulfhydryl groups on proteins containing no free sulfhydryls.

Crosslinking reagents containing an amine-reactive NHS ester on one end and an iodoacetyl group on the other end are particularly useful for two-step protein conjugation. Heterobifunctional reagents like SIAB (Chapter 5, Section 1.5), SIAX (Chapter 5, Section 1.8), or SIAC (Chapter 5, Section 1.9) can be used to modify amine-containing molecules, resulting in iodoacetyl derivatives capable of coupling to sulfhydryl-containing molecules.

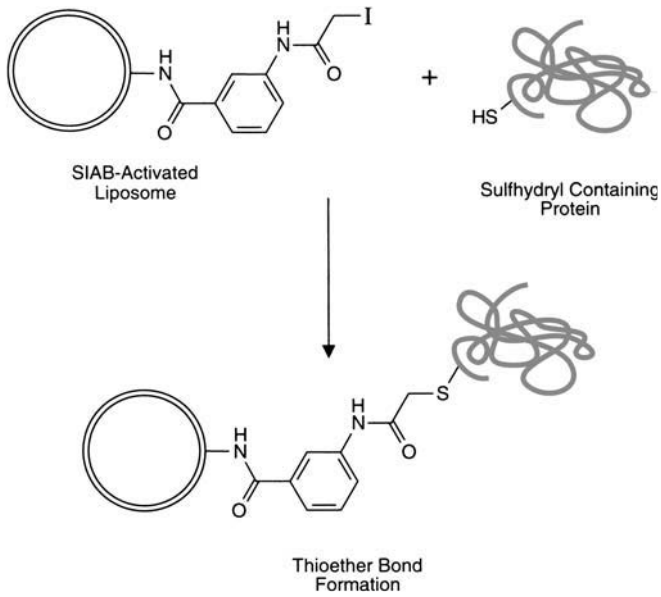


Figure 22.29 SIAB-activated liposomes can couple with sulfhydryl-containing proteins to produce thioether linkages.

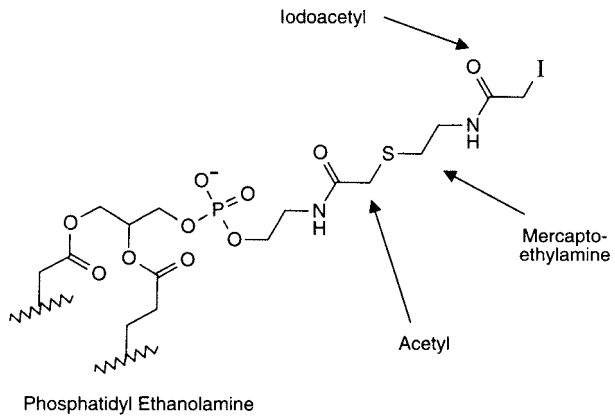


Figure 22.30 An iodoacetamide derivative of PE containing an extended spacer arm can be constructed through a carbodiimide coupling of iodoacetic acid to PE, followed by reaction with 2-mercaptoethylamine, and finally another reaction with iodoacetate.

Liposomes containing PE lipid components may be activated with these crosslinkers to contain iodoacetyl derivatives on their surface (Figure 22.29). The reaction conditions described in Chapter 5, Section 1.5 may be used, substituting a liposome suspension for the initial protein being modified in that protocol. The derivatives are stable enough in aqueous solution to allow purification of the modified vesicles from excess reagent (by dialysis or gel filtration) without

loss of activity. The only consideration is to protect the iodoacetyl derivative from light, which may generate iodine and reduce the activity of the intermediate. Finally, the modified liposome can be mixed with a sulfhydryl-containing molecule to effect the conjugation through a thioether bond.

Alternatively, pure PE may be derivatized to contain iodoacetyl groups prior to vesicle formation. This may be done using heterobifunctional crosslinkers or through the use of iodoacetic anhydride according to Hashimoto *et al.* (1986). However, a single iodoacetyl group on PE was found not to be sufficiently extended from the vesicle surface to allow efficient protein coupling. Only after creating a longer spacer by reacting 2-mercaptoethylamine with the initial iodoacetamide derivative and then reacting a second iodoacetic anhydride to form an extended arm, did the active derivative possess enough length to give it conjugation capability (Figure 22.30). This example illustrates the importance of a long spacer in avoiding steric problems during conjugation to the vesicle surface.

23

Avidin–Biotin Systems

One of the most popular methods of noncovalent conjugation is to make use of the natural strong binding of (strept)avidin for the small molecule biotin. The strength of the (strept)avidin–biotin interaction has made it a useful tool in specific targeting applications and assay design. Since each (strept)avidin molecule contains a maximum of four biotin binding sites, the interaction can be used to enhance the signal strength in immunoassay systems.

Modification reagents that can add a functional biotin group to proteins, nucleic acids, and other molecules now come in many shapes and reactivities (Chapter 11 and Chapter 18, Section 3). Depending on the functionality present on the biotinylation compound, specific reactive groups on antibodies or other proteins may be modified to create a (strept)avidin binding site. Amines, carboxylates, sulfhydryls, and carbohydrate groups can be specifically targeted for biotinylation through the appropriate choice of biotin derivative. In addition, photoreactive biotinylation reagents (Chapter 11, Section 3.4) are used to add nonselectively a biotin group to molecules containing no convenient functional groups for modification. In this manner, oligonucleotide probes often are modified for detection with (strept)avidin conjugates (Chapter 27, Section 2.3).

The following sections discuss the concept and use of the (strept)avidin–biotin interaction in bioconjugate techniques. Preparation of biotinylated molecules and (strept)avidin conjugates also are reviewed with suggested protocols. For a discussion of the major biotinylation reagents, see Chapter 11 and Chapter 18, Section 3.

1. The Avidin–Biotin Interaction

Avidin is a glycoprotein found in egg whites that contains four identical subunits of 16,400 Da each, giving an intact molecular weight of approximately 66,000 (Green, 1975). Each subunit contains one binding site for biotin, or vitamin H, and one oligosaccharide modification (Asn-linked). The tetrameric protein is highly basic, having a pI of about 10. The biotin interaction with avidin is among the strongest noncovalent affinities known, exhibiting a dissociation constant of about 1.3×10^{-15} M. Tryptophan and lysine residues in each subunit are known to be involved in forming the binding pocket (Gitlin *et al.*, 1987, 1988).

The tetrameric native structure of avidin is resistant to denaturation under extreme chaotropic conditions. Even in 8 M urea or 3 M guanidine hydrochloride the protein maintains structural integrity and activity (Green, 1963). When biotin is bound to avidin, the interaction promotes even greater stability to the complex. An avidin–biotin complex (ABC) is resistant to break down in the presence of up to 8 M guanidine at pH 5.2. A minimum of 6–8 M guanidine at pH 1.5 is required for inducing complete dissociation of the avidin–biotin interaction (Cuatrecasas and Wilchek, 1968; Bodanszky and Bodanszky, 1970). Since the subunits in avidin are not held together by disulfide bonds, conditions that cause denaturation also result in subunit disassociation.

The strength of the noncovalent avidin–biotin interaction along with its resistance to break down makes it extraordinarily useful in bioconjugate chemistry. Biotinylated molecules and avidin conjugates can "find" each other under the most extreme conditions to bind and complex together. The biospecificity of the interaction is similar to antibody–antigen or receptor–ligand recognition, but on a much higher level with respect to affinity constants. Variations in buffer salt, pH, the presence of denaturants or detergents, and extremes of temperature will not prevent the interaction from occurring (Ross *et al*., 1986).

The only disadvantage to the use of avidin is its tendency to bind nonspecifically with components other than biotin due to its high pI and carbohydrate content. The strong positive charge on the protein causes ionic interactions with more negatively charged molecules, especially cell surfaces. In addition, carbohydrate binding proteins on cells can interact with the polysaccharide portions on the avidin molecule to bind them in regions devoid of targeted biotinylated molecules. These nonspecific interactions can lead to elevated background signals in some assays, preventing the full potential of the avidin–biotin amplification process to be realized.

Streptavidin is a similar biotin binding protein to avidin, but it is of bacterial origin and originates from *Streptomyces avidinii*. Due to streptavidin's structural differences, however, it can overcome some of the nonspecific binding deficiencies of avidin (Chaiet and Wolf, 1964). Similar to avidin, streptavidin contains four subunits, each with a single biotin binding site. After some post-secretory modifications, the intact tetrameric protein has a molecular mass of about 60,000 Da, slightly less than that of avidin (Bayer *et al*., 1986, 1989).

The primary structure of streptavidin is considerably different than that of avidin, despite the fact that they both bind biotin with similar avidity. This variation in the amino acid sequence results in a much lower isoelectric point for streptavidin (pI 5–6) compared to the highly basic pI of 10 for avidin. Moderation in the overall charge of the protein substantially reduces the amount of nonspecific binding due to ionic interaction with other molecules. Of additional significance is the fact that streptavidin is not a glycoprotein, thus there is no potential for binding to carbohydrate receptors. These factors lead to better signal-to-noise ratios in assays using streptavidin–biotin interactions than those employing avidin–biotin.

Both avidin and streptavidin can be conjugated to other proteins or labeled with various detection reagents without loss of biotin binding activity. Streptavidin is slightly less soluble in water than avidin, but both are extremely robust proteins that can tolerate a wide range of buffer conditions, pH values, and chemical modification processes. Bioconjugate techniques can utilize the ε- or N-terminal amines on these proteins for direct conjugation or employ modification reagents to transform their existing functional groups into other reactive groups (Chapter 1, Section 4).

In the following sections, the use of the term "(strept)avidin" is meant to infer that either avidin or streptavidin can be used in the associated protocols, conjugates, and applications.

2. Use of (Strept)avidin–Biotin Interactions in Assay Systems

The specificity of biotin binding to (strept)avidin provides the basis for developing assay systems to detect or quantify analytes. Biotinylated molecules can be targeted in complex mixtures by using the appropriate (strept)avidin conjugates. If the biotinylated component has affinity for binding a particular antigen, then the antigen can be located through the use of an (strept)avidin conjugate containing a detectable molecule. A series of (strept)avidin–biotin interactions can be built upon each other—utilizing the multivalent nature of each tetrameric (strept)avidin molecule—to further enhance the detection capability for the target.

A common application for (strept)avidin–biotin chemistry is in immunoassays. The specificity of antibody molecules provides the targeting capability to recognize and bind particular antigen molecules. If there are biotin labels on the antibody, it creates multiple sites for the binding of (strept)avidin. If (strept)avidin is in turn labeled with an enzyme, fluorophore, etc., then a very sensitive antigen detection system is created. The potential for more than one labeled (strept)avidin to become attached to each antibody through its multiple biotinylation sites is the key to dramatic increases in assay sensitivity over that obtained through the use of antibodies directly labeled with a detectable tag.

There are several basic immunoassay designs that make use of the enhanced sensitivity afforded by the (strept)avidin–biotin interaction. Most of these assays use conjugates of (strept)avidin with enzymes (such as horseradish peroxidase (HRP) or alkaline phosphatase), although other labels (such as fluorophores) can be used as well. In the simplest assay design, called the labeled avidin–biotin (LAB) system (Figure 23.1), a biotinylated antibody is allowed to incubate and bind with its target antigen. Next, a (strept)avidin–enzyme conjugate is introduced and allowed to interact with the available biotin sites on the bound antibody. Just as in

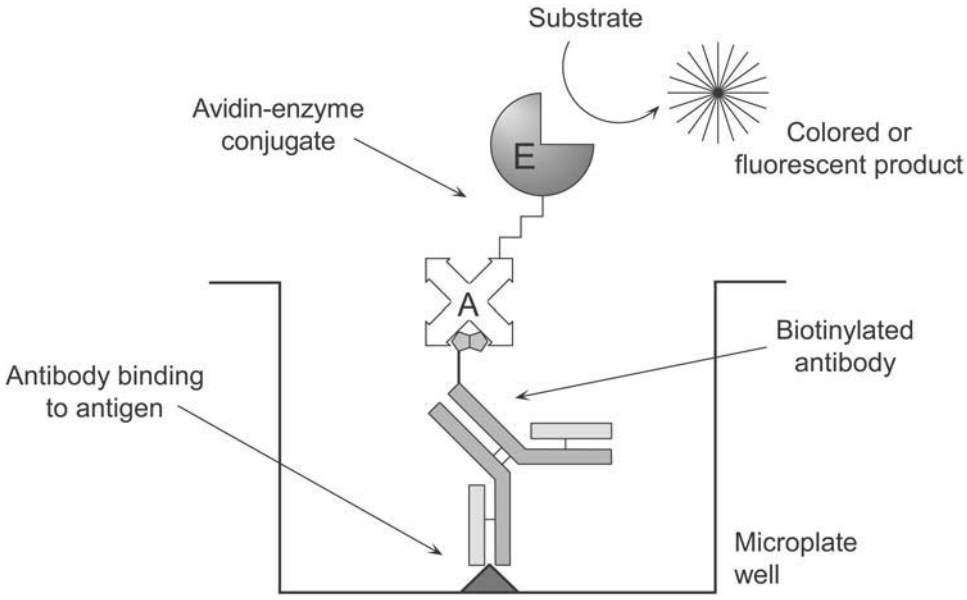


Figure 23.1 The basic design of the LAB assay system.

other enzyme-linked immunosorbent assay (ELISA) tests, substrate development then provides the chemical detectability necessary to quantify the antigen (Guesdon *et al.*, 1979).

In a slightly more complex design, the bridged avidin–biotin (BRAB) system uses (strept) avidin's multiple biotin binding sites to create an assay of potentially higher sensitivity than that of the LAB assay. Again the biotinylated antibody is allowed to bind to its target, but in the next step an unmodified (strept)avidin is introduced to bind with the biotin binding sites on the antibody. Finally, a biotinylated enzyme is added to provide a detection vehicle (Figure 23.2). Since the bound (strept)avidin still has additional biotin binding sites available, the potential exists for more than one biotinylated enzyme to interact with each bound (strept)avidin. In some cases, sensitivity can be increased over that of the LAB technique by using this bridging ability of (strept)avidin.

A modification on this theme can be used to produce one of the most sensitive enzyme-linked assay systems known. The ABC system (for avidin–biotin complex) increases the detectability of antigen beyond that possible with either the LAB or BRAB designs by forming a polymer of biotinylated enzyme and (strept)avidin before its addition to an antigen-bound, biotinylated antibody (Bayer *et al.*, 1988). When (strept)avidin and a biotinylated enzyme are mixed together in solution in the proper proportion, the multiple binding sites on (strept)avidin create a linking matrix to form a high-molecular-weight complex. If the biotinylated enzyme is not in large enough excess to block all the binding sites on (strept)avidin, then additional sites will still be available on this complex to bind a biotinylated antibody which is bound to its complementary antigen. The large complex provides multiple enzyme molecules to enhance the sensitivity of detecting antigen (Figure 23.3). Thus, the ABC procedure is currently among the highest-sensitivity methods available for immunoassay work.

Similar techniques can be used to devise (strept)avidin–biotin assay systems for detection of nucleic acid hybridization. DNA probes labeled with biotin can be detected after they bind

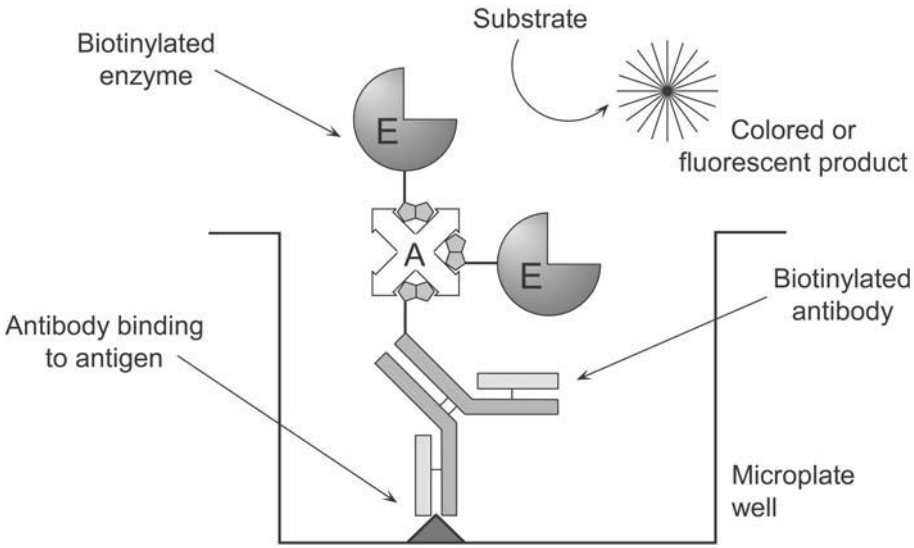


Figure 23.2 The basic design of the BRAB assay system.

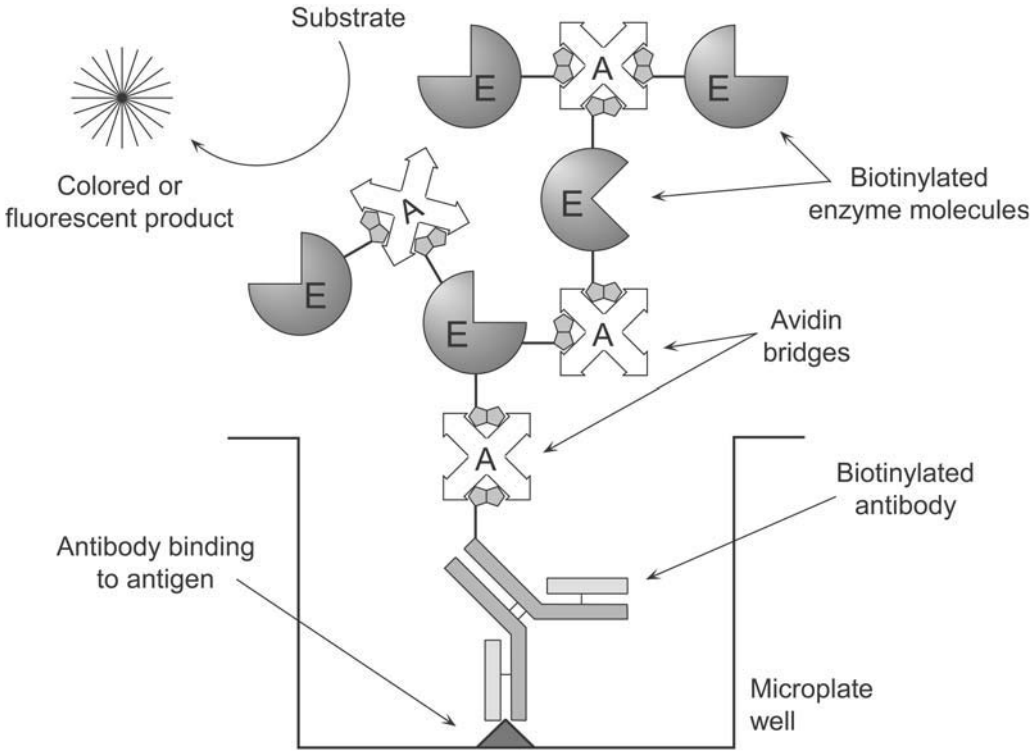


Figure 23.3 The assay design of the ABC system.

their complementary DNA target through the use of (strept)avidin-labeled complexes (Bugawan *et al.*, 1990; Lloyd *et al.*, 1990). Direct detection of hybridized probes can be accomplished, similar to the LAB method, by incubating with an (strept)avidin–enzyme conjugate followed by substrate development. BRAB-like and ABC-like assays also can be utilized to enhance further a DNA probe signal (Chapter 27, Section 2.3).

Non-enzyme assay systems can be designed with the (strept)avidin–biotin interaction, as well. Fluorescently labeled (strept)avidin molecules can be used to detect a biotinylated molecule after it has bound its target. In fact, a single preparation of a fluorescent (strept)avidin derivative can be used as a universal detection reagent for any biotinylated targeting molecule. The main application of this technique is in cytochemical staining wherein the fluorescence signal is used to localize antigen or receptor molecules in cells and tissue sections. In addition, detection of analytes on arrays commonly is done using fluorescently labeled (strept)avidin conjugates to bind to biotinylated primary antibodies interacting with specific targets on the array surface.

Other tags or probes can be coupled to (strept)avidin and used in a similar fashion. For instance, radiolabeled (strept)avidin can be employed as a universal detection reagent in radioimmunoassay designs (Wojchowski and Sytkowski, 1986) (Chapter 12). (Strept)avidin labeled with ^{125}I can be used to localize biotinylated monoclonal antibodies directed against tumor cells *in vivo* for imaging purposes (Paganelli *et al.*, 1988). Chemical tags such as in hydrazide-(strept)avidin derivatives can be made to site-direct (strept)avidin's interaction toward oxidized carbohydrate residues for specific detection of glycoconjugates (Section 5, this chapter).

Colloidal gold-labeled (strept)avidin can be used as highly sensitive detection reagents for microscopy techniques (Cubie and Norval, 1989) (Chapter 24). Finally, cytotoxic substances coupled to (strept)avidin can be used to direct cell-killing activity toward a tumor-cell-bound, biotinylated monoclonal antibody (or other targeting molecule) for cancer therapy (Hashimoto *et al.*, 1984) (Chapter 21).

Universal detection reagents also can be constructed through biotinylation techniques. Modification of immunoglobulin binding proteins with biotin tags, for instance, creates a reagent useful for the general assay of antibody molecules. In this sense, biotinylated protein A or biotinylated protein G can be used to detect the binding of any primary IgG to its antigen target (provided there is no other antibody molecules presence to cause nonspecific binding of the protein A component). Subsequent addition of a labeled (strept)avidin molecule binds to the biotinylated protein A, completing the formation of a detection complex (Jagannath and Sehgal, 1989).

To develop assay systems using the (strept)avidin–biotin interaction, it is first necessary to produce the associated (strept)avidin conjugates and/or biotinylated components. When the LAB technique is employed, the (strept)avidin conjugate is made using crosslinking agents, not biotinylation reagents, in order to maintain the binding capacity of the (strept)avidin tetramer toward other biotinylated molecules. In the BRAB assay system, (strept)avidin is left unconjugated and acts merely as the multivalent bridging molecule, while both the targeting molecule and the detection molecule are biotinylated. The components for the ABC assay are identical to the BRAB system.

The following sections discuss the main techniques used to make (strept)avidin conjugates and various biotinylated components. Chapter 11 and Chapter 18, Section 3 should be consulted for a complete overview of biotinylation reagents.

3. Preparation of (Strept)avidin Conjugates

Conjugates of (strept)avidin with other protein molecules must be prepared to design systems using the LAB assay technique. Suitable protein molecules attached to (strept)avidin either possess indigenous detectability, such as in the case of ferritin or phycobiliproteins, or possess catalytic activity (enzymatic) that can be utilized to produce a detectable substrate product. The majority of conjugation procedures for making (strept)avidin–protein conjugates use the amines, sulfhydryls, or carbohydrates on each protein as functional groups for crosslinking.

Perhaps the most common conjugates of (strept)avidin involve attaching enzyme molecules for use in ELISA systems. As in the case of antibody–enzyme conjugation schemes (Chapter 20), by far the most commonly used enzymes for this purpose are HRP and alkaline phosphatase. Other enzymes such as β-galactosidase and glucose oxidase are used less often, especially with regard to assay tests for clinically important analytes (Chapter 26).

Other proteins commonly crosslinked to (strept)avidin are chromogenic or fluorescent molecules, such as ferritin or phycobiliproteins (Chapter 9, Section 7). These conjugates can be used in microscopy techniques to stain and localize certain antigens or receptors in cells or tissue sections.

The following sections discuss three main methods for preparing these types of (strept)avidin–protein conjugates. They involve using an *N*-hydroxysuccinimide (NHS) ester–maleimide heterobifunctional crosslinker, making use of the carbohydrate on glycoproteins for reductive amination coupling, and employing the old technique of homobifunctional crosslinking with glutaraldehyde.

3.1. NHS Ester–Maleimide-Mediated Conjugation Protocols

Heterobifunctional crosslinking agents can be used to control the degree of protein conjugation, thus limiting polymerization and controlling the molar ratio of each component in the final complex (Chapter 5). Particularly useful heterobifunctionals include the amine- and sulfhydryl-reactive NHS ester–maleimide crosslinkers discussed in Chapter 5, Section 1. Chief among these is succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) or sulfo-SMCC (Chapter 5, Section 1.3), which contains a reasonably long spacer and an extremely stable maleimide group due to the adjacent cyclohexane ring in its cross-bridge.

Conjugations done with SMCC usually involve up to three steps. In the first stage, one of the proteins is modified at its amine groups via the NHS ester end of the crosslinker to form amide linkages, which upon modification then create derivatives that terminate in reactive maleimide groups. If the other protein to be conjugated does not contain sulfhydryl residues necessary to react with the maleimide-activated protein, it must be modified to contain them (Chapter 1, Section 4.1). Finally, the two reactive components are mixed together in the proper ratio to effect the conjugation reaction.

For the preparation of (strept)avidin–enzyme conjugates, either protein may be first modified with SMCC and the other one modified to contain —SH groups. Since (strept)avidin does not possess any free sulfhydryls—and the disulfides present in (strept)avidin are inaccessible to easy reduction—it must be modified with either a crosslinker or with a thiolating agent before conjugation. If the enzyme employed contains free sulfhydryls in its native state, such as β-galactosidase, then it is convenient to activate (strept)avidin with SMCC and simply add the sulfhydryl-containing protein to it for conjugation. If the enzyme does not contain free sulfhydryls (as is the case with alkaline phosphatase or HRP), then the choice of which component gets maleimide activated and which gets thiolated is up to the individual.

The following protocol describes the activation of (strept)avidin with sulfo-SMCC and its subsequent conjugation with an enzyme modified to contain sulfhydryls using *N*-succinimidyl-*S*-acetylthioacetate (SATA) (Chapter 1, Section 4.1). A method for the opposite approach, wherein the enzyme is activated with SMCC and the (strept)avidin component is thiolated, is presented immediately after this protocol. This strategy may be the most common approach to forming these conjugates (Figure 23.4). In addition, since there are enzymes commercially available that are preactivated with SMCC (Thermo Fisher), their use may be the easiest solution to forming conjugates.

Protocol for the Conjugation of SMCC-Activated (Strept)avidin with Thiolated Enzyme

Activation of (Strept)avidin with SMCC

1. Dissolve (strept)avidin (Thermo Fisher) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Add 1.0 mg of sulfo-SMCC (Thermo Fisher) to each ml of (strept)avidin solution. Mix to dissolve.
3. React for 30–60 minutes at room temperature. Since maleimide groups are labile in aqueous solution, extended reaction times should be avoided.
4. Immediately purify the maleimide-activated (strept)avidin away from excess crosslinker and reaction by-products by gel filtration on a desalting resin. A spin column will facilitate the

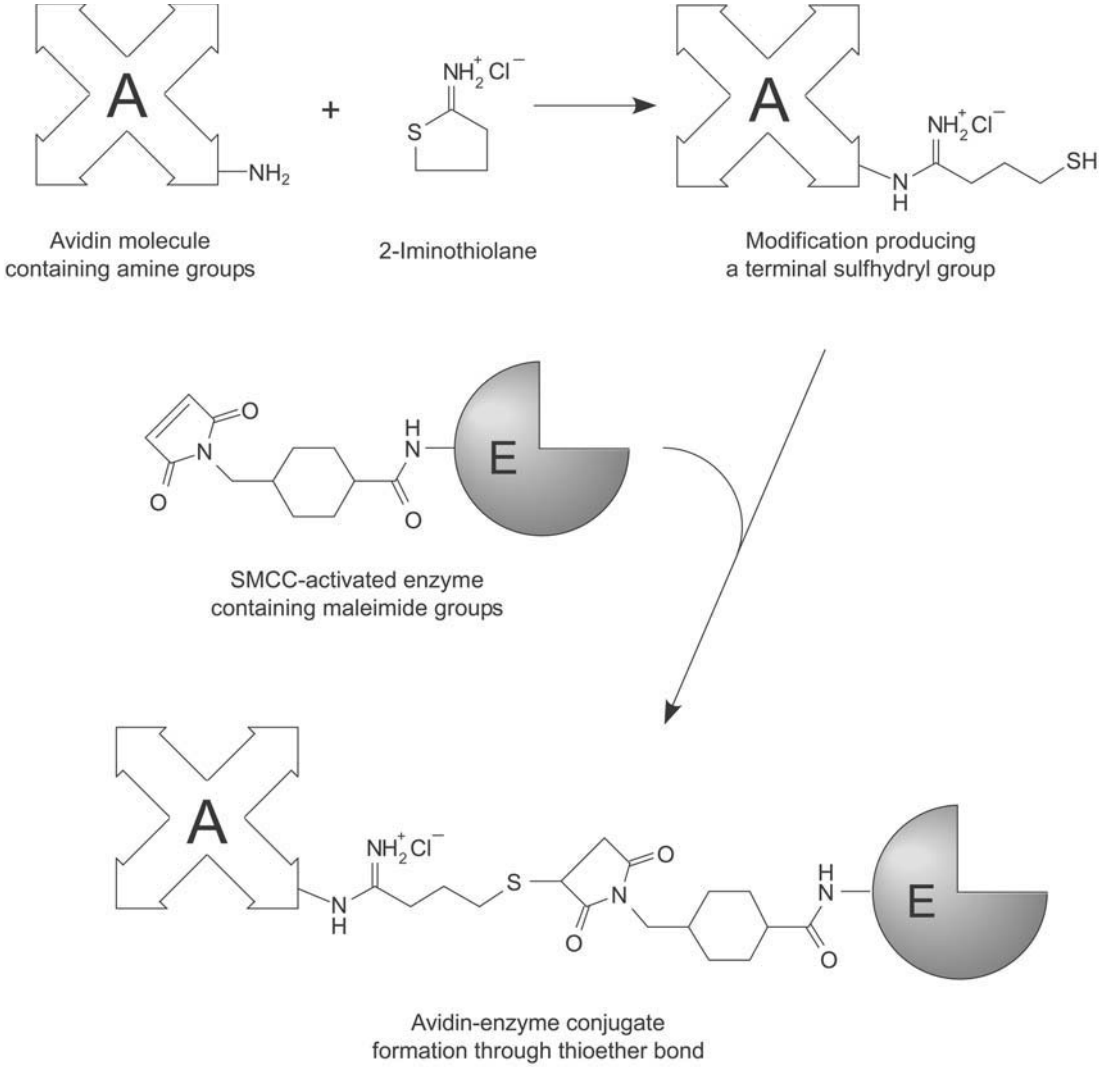


Figure 23.4 Avidin may be modified with 2-iminothiolane to produce sulfhydryl groups. Subsequent reaction with a maleimide-activated enzyme produces a thioether-linked conjugate.

most rapid purification. Use 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, as the chromatography buffer. Pool the fractions containing protein (the first peak eluting from the column). After elution, adjust the protein concentration to 10 mg/ml for the conjugation reaction (centrifugal concentrators work well for this step). At this point, the maleimide-activated (strept)avidin may be frozen and lyophilized to preserve its maleimide activity. The modified protein is stable for at least 1 year in a freeze-dried state. If kept in solution, the maleimide-activated (strept)avidin is labile and should be used immediately to conjugate with a thiolated enzyme following the procedure described below.

Modification of Enzyme with SATA

If β-galactosidase is used to conjugate with an SMCC-activated (strept)avidin, then there is no need to thiolate the enzyme, since it contains sulfhydryls in its native state (Fujiwara *et al.*, 1988; Sivakoff and Janes, 1988). For conjugations using HRP, alkaline phosphatase, or glucose oxidase, however, thiolation is necessary to add the requisite sulfhydryls.

1. Dissolve the enzyme to be modified in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Prepare a stock solution of SATA (Thermo Fisher) by dissolving it in DMSO at a concentration of 13 mg/ml. Use a fume hood to handle the organic solvent.
3. Add 25 µl of the SATA stock solution to each ml of 10 mg/ml enzyme solution. For different concentrations of enzyme in the reaction medium, proportionally adjust the amount of SATA addition; however do not exceed 10 percent DMSO in the aqueous reaction medium.
4. React for 30 minutes at room temperature.
5. To purify the SATA-modified enzyme perform a gel filtration separation using a desalting resin or dialyze against 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA. Purification is not absolutely required, since the following deprotection step is done using hydroxylamine at a significant molar excess over the initial amount of SATA added. Whether a purification step is done or not, at this point, the derivative is stable and may be stored under conditions which favor long-term enzyme activity.
6. Deprotect the acetylated sulfhydryl groups on the SATA-modified enzyme according to the following protocol:
   a. Prepare a 0.5 M hydroxylamine solution in 0.1 M sodium phosphate, pH 7.2, containing 10 mM EDTA.
   b. Add 100 µl of the hydroxylamine stock solution to each ml of the SATA-modified enzyme. Final concentration of hydroxylamine in the enzyme solution is 50 mM.
   c. React for 2 hours at room temperature.
   d. Purify the thiolated enzyme by gel filtration on a desalting resin using 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.2, containing 10 mM EDTA as the chromatography buffer. To obtain efficient separation between the thiolated protein and excess hydroxylamine and reaction by-products, the sample size applied to the column should be at a ratio of no more than 5 percent sample volume to the total column volume. Collect 0.5 ml fractions. Pool the fractions containing protein by measuring the absorbance of each fraction at 280 nm.

Production of Conjugate

1. Immediately mix the thiolated enzyme with an amount of maleimide-activated (strept) avidin to obtain the desired molar ratio of enzyme-to-(strept)avidin in the conjugate. Use of a 4:1 (enzyme:avidin) molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures employing the LAB approach.
2. React for 30–60 minutes at 37°C or 2 hours at room temperature. The conjugation reaction also may be done at 4°C overnight.

A variation of the above method can be used, wherein the enzyme is first activated with SMCC and conjugated to a thiolated (strept)avidin molecule. This approach probably is the most common way of preparing (strept)avidin–enzyme conjugates, and since the preactivated enzymes are readily available (Thermo Fisher), it also may be the easiest.

Protocol for the Conjugation of SMCC-Activated Enzymes with Thiolated (Strept)avidin

Activation of Enzyme with Sulfo-SMCC

The following protocol describes the activation of HRP with sulfo-SMCC. Other enzymes may be activated in a similar manner. The activated enzyme possesses maleimide groups that are relatively unstable in aqueous solution. Therefore, the thiolation reaction should be coordinated with the activation process so that the final conjugation can be done immediately. *Note*: If preactivated enzymes are obtained (Thermo Fisher), this step may be eliminated.

1. Dissolve HRP in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Add 3.3 mg of sulfo-SMCC (Thermo Fisher) to each ml of the HRP solution. Mix to dissolve and react for 30 minutes at room temperature. Alternatively, two equal additions of crosslinker may be done—the second one after 15 minutes of incubation—to obtain even more efficient modification.
3. Immediately purify the maleimide-activated HRP away from excess crosslinker and reaction by-products by gel filtration on a desalting column. Use 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, as the chromatography buffer. HRP can be observed visually as it flows through the column due to the color of its heme ring. Pool the fractions containing the HRP peak. After elution, adjust the HRP concentration to 10 mg/ml for the conjugation reaction. At this point, the maleimide-activated enzyme may be frozen and lyophilized to preserve its maleimide activity. The modified enzyme is stable for at least 1 year in a freeze-dried state. If kept in solution, the maleimide-activated HRP should be used immediately to conjugate with thiolated (strept)avidin following the protocols outlined below.

Thiolation of (Strept)avidin

1. Dissolve (strept)avidin in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10 mg/ml.
2. Prepare a stock solution of SATA by dissolving it in DMSO at a concentration of 13 mg/ml. Use a fume hood to handle the organic solvent.
3. Add 25 µl of the SATA stock solution to each ml of 10 mg/ml (strept)avidin solution. For different concentrations of protein in the reaction medium, proportionally adjust the amount of SATA addition; however do not exceed 10 percent DMSO in the aqueous reaction medium.
4. React for 30 minutes at room temperature.
5. To purify the SATA-modified (strept)avidin use gel filtration on a desalting column or dialyze against 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, containing 10 mM EDTA. At this point, the derivative is stable and may be stored under conditions which favor long-term (strept)avidin activity.

6. Deprotect the acetylated sulfhydryl groups on the SATA-modified protein according to the following protocol:
   a. Prepare a 0.5 M hydroxylamine solution in 0.1 M sodium phosphate, pH 7.2, containing 10 mM EDTA.
   b. Add 100 µl of the hydroxylamine stock solution to each ml of the SATA-modified (strept)avidin. Final concentration of hydroxylamine in the solution is 50 mM.
   c. React for 2 hours at room temperature.
   d. Purify the thiolated protein by gel filtration on Sephadex G-25 using 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.2, containing 10 mM EDTA as the chromatography buffer.

Conjugation of SMCC-Activated Enzyme with Thiolated (Strept)avidin

1. Immediately mix the SMCC-activated enzyme with an amount of thiolated (strept)avidin to obtain the desired molar ratio of enzyme-to-(strept)avidin in the conjugate. Use of a 4:1 (enzyme:avidin) molar ratio in the conjugation reaction usually results in high-activity conjugates suitable for use in many enzyme-linked immunoassay procedures employing the LAB approach.
2. React for 30–60 minutes at 37°C or 2 hours at room temperature. The conjugation reaction also may be done at 4°C overnight.

3.2. Conjugation Using Periodate Oxidation/Reductive Amination

Glycoproteins may be conjugated with another amine-containing protein through the process of periodate oxidation and reductive amination. Periodate oxidation of polysaccharide components on the glycoprotein results in the formation of reactive aldehyde residues by cleavage of carbon–carbon bonds and oxidation of the associated adjacent hydroxyls (Chapter 1, Section 4.4). Conjugation with another protein may be done by reacting the aldehydes with amines to form intermediate Schiff bases with subsequent reduction using sodium cyanoborohydride to create stable secondary amine bonds.

This method of conjugation is particularly well suited for coupling HRP or ferritin with (strept)avidin. Both HRP and (strept)avidin are glycoproteins that can be oxidized with sodium periodate to generate aldehydes. Thus, HRP–(strept)avidin and ferritin–(strept)avidin may be prepared by reductive amination. Ferritin is a large, complex protein of molecular weight 750,000. Its structure is made of a protein shell of diameter approximately 12 nm that surrounds a micelle core consisting of ferric hydroxide of about 6 nm in diameter. This core contains more than 2,000 iron atoms, making the protein extremely electron dense and thus perfect for electron microscopy applications. The properties of HRP are described in Chapter 26, Section 1.

The following protocol is adapted from Bayer *et al*. (1976).

Protocol for the Conjugation of (Strept)avidin with Ferritin Using Reductive Amination

1. Dissolve (strept)avidin in 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5, at a concentration of 3 mg/ml.
2. Dissolve ferritin in 0.1 M sodium acetate, 0.15 M NaCl, pH 4.5, at a concentration of 100 mg/ml.

3. Add 1 ml of ferritin solution to every 5 ml of (strept)avidin solution. Chill on ice.
4. Dissolve sodium periodate in water at a concentration of 100 mM. Prepare fresh and protect from light.
5. Add 110 µl of sodium periodate solution to each ml of (strept)avidin/ferritin solution.
6. React for 3 hours on ice with periodic mixing. Protect from light.
7. Remove excess periodate by gel filtration on a column of Sephadex G-25 or by overnight dialysis against 50 mM sodium borate, 0.15 M NaCl, pH 8.5.
8. Dissolve 10 mg of sodium borohydride in 1 ml of 10 mM NaOH. Prepare fresh. Add 83 µl of this reducing solution to each ml of (strept)avidin/ferritin solution.
9. React for 1 hour on ice.
10. Remove excess reductant by gel filtration using a column of Sephadex G-25 or by extensive dialysis against 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4.

Conjugation of HRP by reductive amination can be done by oxidizing the carbohydrate on the enzyme and subsequently coupling to the amines on (strept)avidin (Figure 23.5).

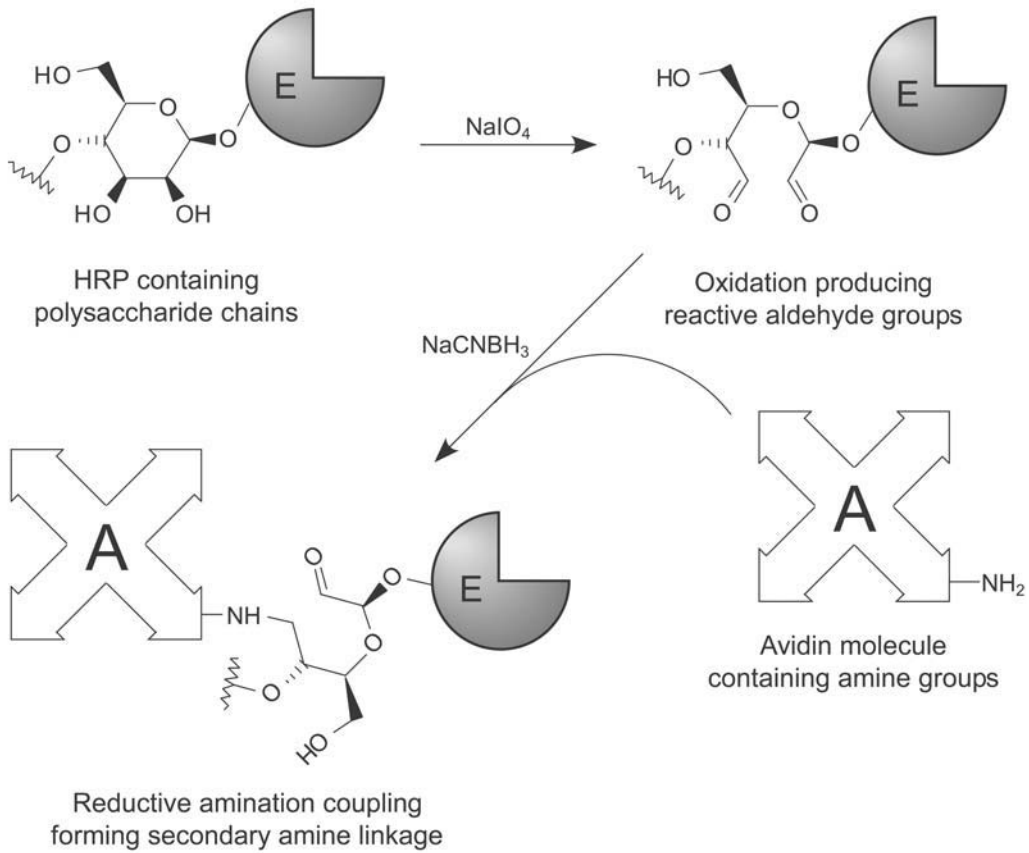


Figure 23.5 Oxidation of the polysaccharide components of HRP produces reactive aldehyde groups. Conjugation to avidin then may be done by reductive amination.

Protocol for the Preparation of (Strept)avidin–HRP by Reductive Amination

Oxidation of HRP with Sodium Periodate

1. Dissolve HRP in water or 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10–20 mg/ml.
2. Dissolve sodium periodate in water at a concentration of 0.088 M. Protect from light.
3. Immediately add 100 µl of the sodium periodate solution to each ml of the HRP solution. This results in a 8 mM periodate concentration in the reaction mixture. Mix to dissolve. Protect from light.
4. React in the dark for 15 minutes at room temperature. A color change will be apparent as the reaction proceeds—changing from the brownish/gold color of concentrated HRP to green. Longer reaction times will result in a decrease in HRP enzymatic activity.
5. Immediately purify the oxidized enzyme by gel filtration using a column of Sephadex G-25. The chromatography buffer is 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2. Collect 0.5 ml fractions and monitor for protein at 280 nm. HRP also may be detected by its absorbance at 403 nm. In oxidizing large quantities of HRP, the fraction collection process may be done visually—just pooling the colored HRP peak as it comes off the column.
6. Pool the fractions containing protein. Adjust the enzyme concentration to 10 mg/ml for the conjugation step. The periodate-activated HRP may be stored frozen or freeze-dried for extended periods without loss of activity. However, do not store the preparation in solution at room temperature or 4°C, since precipitation will occur over time due to self-polymerization.

Conjugation of Periodate-Oxidized HRP with (Strept)avidin

1. Dissolve (strept)avidin at a concentration of 10 mg/ml in 0.2 M sodium bicarbonate, pH 9.6, at room temperature. The high-pH buffer will result in very efficient Schiff base formation and conjugation with the highest possible incorporation of enzyme molecules per (strept)avidin molecule. To produce lower-molecular-weight conjugates (using less efficient Schiff base formation conditions), dissolve the proteins at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2.
2. The periodate-oxidized HRP (prepared above) is finally purified using 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2. For conjugation using the lower-pH buffered environment, this HRP preparation can be used directly at 10 mg/ml concentration. For conjugation using the higher-pH carbonate buffer, dialyze the HRP solution against 0.2 M sodium carbonate, pH 9.6 for 2 hours at room temperature prior to use.
3. Mix the (strept)avidin solution with the enzyme solution at a ratio of 1:6.6 (v/v). Since (strept)avidin has a molecular weight of about 66,000 and HRP's molecular weight is 40,000, this ratio of volumes will result in a molar ratio of HRP:(strept)avidin equal to 4:1. For conjugates consisting of greater enzyme-to-(strept)avidin ratios, proportionally increase the amount of enzyme solution as required. Typically, molar ratios of 2:1 to 10:1 (enzyme: avidin) give acceptable conjugates useful in a variety of ELISA techniques.
4. React for 2 hours at room temperature to form the initial Schiff base interactions.
5. In a fume hood, add 10 µl of 5 M sodium cyanoborohydride (Sigma) per ml of reaction solution. *Caution*: Cyanoborohydride is extremely toxic. All operations should be done with care in a fume hood. Also, avoid any contact with the reagent, as the 5 M solution is prepared in 1 N NaOH.

6. React for 30 minutes at room temperature (in a fume hood).
7. Block unreacted aldehyde sites by addition of 50 µl of 1 M ethanolamine, pH 9.6, per ml of conjugation solution. Approximately a 1 M ethanolamine solution may be prepared by addition of 300 µl ethanolamine to 5 ml of deionized water. Adjust the pH of the ethanolamine solution by addition of concentrated HCl, keeping the solution cool on ice.
8. React for 30 minutes at room temperature.
9. Purify the conjugate from excess reactants by dialysis or gel filtration using Sephadex G-25. Use 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.0, as the buffer for either operation. Use a fume hood, since cyanoborohydride will be present in some of the fractions.

3.3. Glutaraldehyde Conjugation Protocol

Glutaraldehyde is one of the oldest homobifunctional reagents used for protein conjugation. It reacts with amine groups to create crosslinks by one of several routes (Chapter 4, Section 6.2). Under reducing conditions, the aldehydes on both ends of glutaraldehyde will couple with amines to form secondary amine linkages. The reagent is highly efficient at protein conjugation, but has a tendency to form high-molecular-weight polymers due to its homobifunctional nature. Single-step protocols using glutaraldehyde are particularly notorious at resulting in some degree of insoluble protein oligomers (Porstmann *et al*., 1985). Two-step methods somewhat alleviate this problem, but the potential for conjugate precipitation is still present.

Preparation of (strept)avidin conjugates with other proteins can be accomplished using either a one- or two-step glutaraldehyde procedure. Both methods may result in some degree of oligomer formation; however, the two-step protocol may keep insoluble material to a minimum. Although the following procedures are described using particular proteins, they may be used as a general guide for coupling enzymes, ferritin, phycobiliproteins, or other detectable proteins to (strept)avidin. Some optimization may be necessary to obtain the best yield of active conjugate.

Protocol for the One-Step Glutaraldehyde Conjugation of Ferritin to (Strept)avidin

This protocol is adapted from Bayer and Wilchek (1980).

1. Prepare a solution containing 5 mg/ml (strept)avidin and 25 mg/ml ferritin in 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, at room temperature. *Note*: For the coupling of other proteins to (strept)avidin, their concentration may be reduced from the 25 mg/ml stated for ferritin.
2. In a fume hood, add 10 µl of 25 percent glutaraldehyde per ml of (strept)avidin/ferritin solution. Mix well.
3. React for 1 hour at room temperature.
4. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.
   Note: Some protocols do not call for a reduction step. The addition of borohydride at this level may result in disulfide bond cleavage and loss of protein activity in some cases. As an alternative to reduction, add 50 µl of 0.2 M lysine in 0.5 M sodium carbonate, pH 9.5 to each ml of the conjugation reaction to block excess reactive sites. Block for 2 hours at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.

5. Reduce for 1 hour at 4°C.
6. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45 µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

A two-step glutaraldehyde protocol may result in lower-molecular-weight conjugates, thus limiting the degree of insoluble material formed during the crosslinking process. The following protocol is adapted from Avrameas (1969).

Protocol for the Two-Step Glutaraldehyde Conjugation of Enzymes to (Strept)avidin

1. Dissolve the enzyme at a concentration of 10 mg/ml in 0.1 M sodium phosphate, 0.15 M NaCl, pH 6.8.
2. Add glutaraldehyde to a final concentration of 1.25 percent.
3. React overnight at room temperature.
4. Purify the activated enzyme from excess glutaraldehyde by gel filtration (using Sephadex G-25) or by dialysis against PBS, pH 6.8.
5. Dissolve (strept)avidin at a concentration of 10 mg/ml in 0.5 M sodium carbonate, pH 9.5. Mix the activated enzyme with the (strept)avidin solution at the desired molar ratio to effect the conjugation. Mixing the equivalent of 1–2 moles of enzyme per mole of (strept)avidin usually results in acceptable conjugates.
6. React overnight at 4°C.
7. To reduce the resultant Schiff bases and any excess aldehydes, add sodium borohydride to a final concentration of 10 mg/ml.
   Note: Some protocols avoid a reduction step, as it can lead to disulfide bond cleavage and detrimental effects on protein activity. As an alternative to reduction, add 50 µl of 0.2 M lysine in 0.5 M sodium carbonate, pH 9.5 to each ml of the conjugation reaction to block excess reactive sites. Block for 2 hours at room temperature. Other amine-containing small molecules may be substituted for lysine—such as glycine, Tris buffer, or ethanolamine.
8. Reduce for 1 hour at 4°C.
9. To remove any insoluble polymers that may have formed, centrifuge the conjugate or filter it through a 0.45 µm filter. Purify the conjugate by gel filtration or dialysis using PBS, pH 7.4.

4. Preparation of Fluorescently Labeled (Strept)avidin

Fluorophore modification of (strept)avidin creates a reagent system that can be used to detect and localize biotinylated targeting molecules. The application of such reagents in immunohistochemical staining techniques is significant (Bonnard *et al.*, 1984). A biotinylated antibody directed against a particular tissue antigen can be allowed to bind its target *in situ*, and then a fluorescently tagged (strept)avidin may be added to bind and visualize the antibody-bound antigenic sites by luminescence. Individual cellular structures can be labeled in similar assay strategies and detected by fluorescent microscopy or cell sorting techniques (Sternberger, 1986; Abou-Samra *et al.*, 1990). Biotinylated targeting molecules like antibodies usually possess low nonspecific binding potential despite the presence of a biotin tag. If hydrophilic biotinylation compounds are used, such as those containing a PEG spacer (Chapter 18), the degree of

nonspecific binding can be kept to a minimum. The multivalent nature of (strept)avidin's biotin binding sites combined with the potential of more than one biotin tag per antibody creates a system of much greater potential sensitivity than when using fluorescently modified antibodies directly. The complex formed from the (strept)avidin–biotin interaction amplifies the fluorescent signal beyond that capable in standard labeled antibody techniques.

Double-labeling systems also can be developed using the (strept)avidin–biotin interaction. If two primary antibodies directed against separate antigenic determinants are labeled, one with biotin and the other with another detection component (such as a fluorophore, enzyme, gold particles, etc.), then both may be used to simultaneously localize different antigens in tissue sections. The biotinylated antibody may be subsequently detected by the addition of a fluorescently labeled (strept)avidin reagent. An example of a double label (strept)avidin–biotin detection system is that of Feller *et al.* (1983). A pair of tonsil antigens was visualized using two monoclonal antibodies, one fluorescein labeled and the other biotinylated. The biotinylated antibody was detected by using a phycoerythrin-labeled (strept)avidin conjugate (Section 4.4, this chapter, and Chapter 9, Section 7). Even triple-labeling systems may be developed using this strategy (van Dongen *et al.*, 1985).

The following sections present suggested protocols for labeling (strept)avidin with selected fluorophores. Other fluorescent probes may be constructed using the reagents and methods discussed in Chapter 9.

4.1. Modification with FITC

Fluorescein isothiocyanate (FITC) has been one of the most common fluorescent labels used to modify proteins and other biomolecules (Chapter 9, Section 1). The isothiocyanate group reacts with amines in protein molecules to form a stable thiourea linkage (Figure 23.6). (Strept)avidin may be tagged with this reagent to yield highly fluorescent derivatives useful both in single-staining and double-staining techniques (Bayer and Wilchek, 1980; Bakkus *et al.*, 1989; Szabo *et al.*, 1989). Optimal modification levels for fluorescein are in the range of 3–8 fluorophores per (strept)avidin molecule. Lower incorporation levels will result in low luminescence and poor sensitivity. Higher levels may cause fluorescein–fluorescein quenching effects, resulting in decreased fluorescence. Too high a modification level also may result in nonspecific binding of the derivatized proteins to nontargeted components in assay systems.

Although FITC and other reactive fluorescein derivatives still are widely used to label (strept)avidin and other proteins, better fluorescence yield and stability will be obtained if one of the newer hydrophilic fluorescein dyes is used. See Chapter 9, Section 1, for additional details on labeling proteins with fluorescein.

Protocol

1. Dissolve (strept)avidin in 0.1 M sodium carbonate, pH 9.5, at a concentration of 2–4 mg/ml.
2. Dissolve FITC in DMF at a concentration of 2 mg/ml. Protect from light.
3. Add 50–100 µl of the FITC solution to each ml of the (strept)avidin solution.
4. React overnight at 4°C in the dark.
5. Remove excess fluorescein by gel filtration using a column of Sephadex G-25.

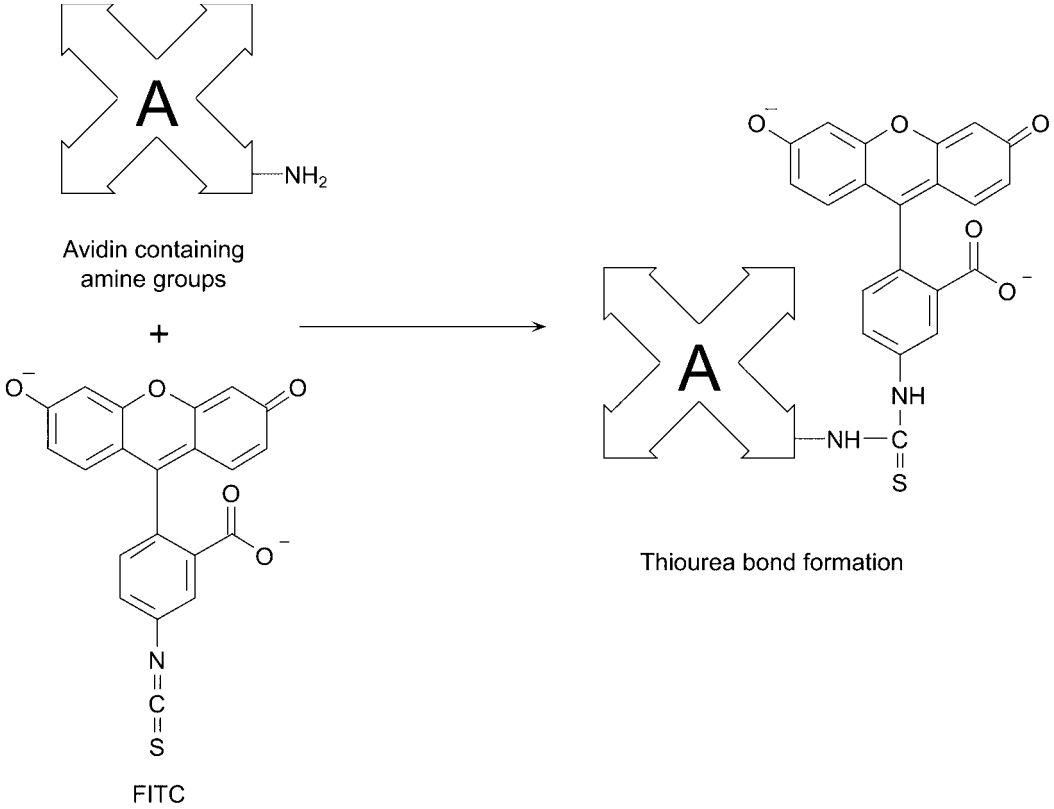


Figure 23.6 The reaction of FITC with avidin produces a fluorescent probe via isothiourea bonds.

4.2. Modification with Lissamine Rhodamine B Sulfonyl Chloride

Rhodamine derivatives are popular probes to use in tandem with fluorescein labels. The Lissamine derivatives of rhodamine (Chapter 9, Section 2) are intensely fluorescent, strongly emitting in the red region of the spectrum. The red luminescence of Lissamine rhodamine contrasts sharply with the green emission of fluorescein derivatives. Lissamine rhodamine B sulfonyl chloride can be used to modify proteins at their ε- and N-terminal amine functional groups. The resultant derivatives are linked through stable sulfonamide bonds, resulting in rhodamine's fluorescent character being incorporated into the modified molecules. (Strept)avidin derivatives of this fluorophore are particularly popular for use in fluorescent assay systems (Figure 23.7).

Protocol

1. Dissolve (strept)avidin in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.0, at a concentration of 1–5 mg/ml.
2. Dissolve Lissamine rhodamine B sulfonyl chloride in DMF at a concentration of 1–2 mg/ml. Protect from light and use immediately. Do not use DMSO as the solvent, as sulfonyl chlorides react with it.

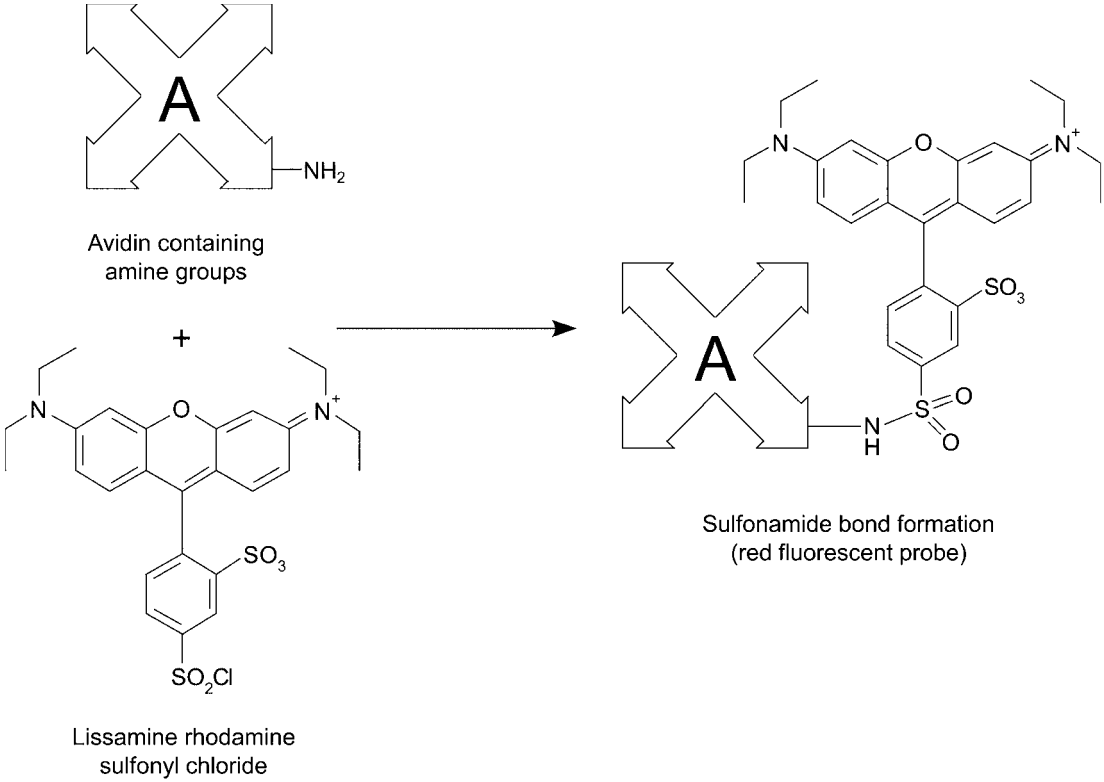


Figure 23.7 Avidin (or (strept)avidin) can be labeled with Lissamine rhodamine sulfonyl chloride to form a fluorescent probe.

3. In a darkened lab and with gentle mixing, slowly add 50–100 µl of the fluorophore solution to each ml of the (strept)avidin solution.
4. React for 1 hour at room temperature in the dark.
5. Remove excess fluorophore by gel filtration using a column of Sephadex G-25 or by dialysis.

Modification of (strept)avidin with Texas Red sulfonyl chloride may be done similarly, except the fluorophore is first dissolved in acetonitrile prior to addition to the aqueous reaction mixture.

4.3. Modification with AMCA–NHS

AMCA derivatives possess fluorescent properties within the blue region of the visible spectrum (Chapter 9, Section 3). Their emission range is well removed from other common fluorophores, making them excellent choices for use in double-labeling techniques, for example with fluorescein-labeled molecules. Coumarin-based fluorescent probes are very good donors for excited-state energy transfer to fluoresceins. AMCA–NHS reacts with amine-containing molecules to result in stable amide bond derivatives (Figure 23.8). (Strept)avidin may be labeled with this reagent to

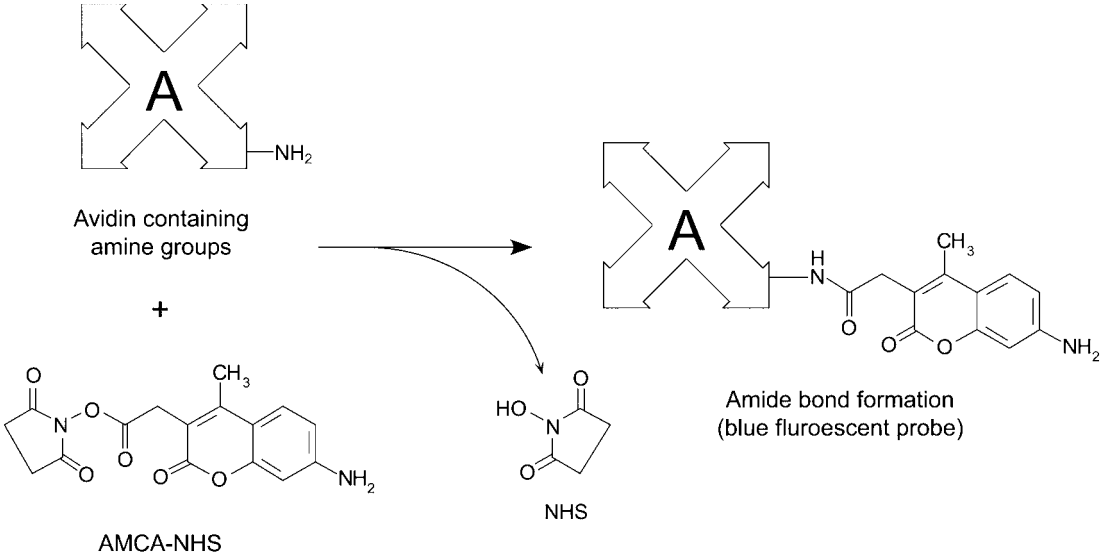


Figure 23.8 AMCA–NHS reacts with the amine groups of avidin (or (strept)avidin) to produce amide bonds.

give probes useful for immunohistochemical staining of biotinylated targeting molecules. AMCA-labeled proteins are fairly stable to photoquenching and exhibit a large Stokes shift, allowing sensitive measurements to be made without interference from scattered excitation light.

Protocol

1. Dissolve (strept)avidin) in 50 mM sodium borate, pH 8.5, at a concentration of 10 mg/ml. Other buffers may be used for an NHS ester reaction, including 0.1 M sodium phosphate, pH 7.5 (Chapter 2, Section 1.4).
2. Dissolve AMCA–NHS (Thermo Fisher) in DMSO at a concentration of 2.6 mg/ml. Protect from light.
3. In subdued lighting conditions, slowly add 50–100 µl of the AMCA–NHS stock solution to each ml of the (strept)avidin solution, with gentle mixing.
4. React for 1 hour at room temperature in the dark.
5. Remove excess reagent and reaction by-products by gel filtration using a column of Sephadex G-25 or by dialysis.

4.4. Conjugation with Phycobiliproteins

Phycobiliproteins are incredibly fluorescent due to their multiple chromophoric bilin prosthetic groups, conferring extremely high absorbance coefficients to each protein molecule (Chapter 9, Section 7). Conjugates of these biliproteins with targeting molecules form extraordinarily luminescent probes. Labeling with phycobiliprotein derivatives can provide absorption coefficients 30-fold higher than labeling with small, synthetic fluorophores. Their ability to be monitored by fluorescing in the red region of the spectrum decreases potential interferences from indigenous biological fluorescence. Phycoerythrin-labeled (strept)avidin probes can be used in

double-staining procedures with a fluorescein-labeled antibody, detecting two antigens in the same tissue section simultaneously by excitation at 488 nm (Feller *et al*., 1983).

The bilin content of these fluorescent proteins ranges from a low of 4 prosthetic groups in C-phycocyanin to the 34 groups of B- and R-phycoerythrin. Phycoerythrin derivatives, therefore, can be used to create the most intensely fluorescent probes possible using these proteins. (Strept)avidin–phycoerythrin conjugates, for example, have been used to detect as little as 100 biotinylated antibodies bound to receptor proteins per cell (Zola *et al*., 1990).

Conjugates of (strept)avidin with these fluorescent probes may be prepared by activation of the phycobiliprotein with N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) to create a sulfhydryl-reactive derivative, followed by modification of (strept)avidin with 2-iminothiolane or SATA (Chapter 1, Section 4.1) to create the free sulfhydryl groups necessary for conjugation. The protocol for SATA modification of (strept)avidin can be found in Section 3.1, this chapter. The procedure for SPDP activation of phycobiliproteins can be found in Chapter 9, Section 7. Reacting the SPDP-activated phycobiliprotein with thiol-labeled (strept)avidin at a molar ratio of 2:1 will result in highly fluorescent biotin binding probes.

Other fluorescent probes also may be used to label (strept)avidin molecules for detection of biotinylated targeting molecules. Chapter 9 reviews many additional fluorescent labels, such as quantum dots, lanthanide chelates, and cyanine dye derivatives, all of which may be used in similar protocols to create detection conjugates for (strept)avidin–biotin-based assays.

5. Preparation of Hydrazide-Activated (Strept)avidin

Hydrazide groups can react with aldehydes or ketones to form hydrazone linkages (Chapter 2, Section 5.1). Proteins may be labeled with hydrazide residues by reaction of their indigenous carboxylate groups with *bis*-hydrazine compounds such as adipic acid dihydrazide or carbohydrazide (Chapter 4, Section 8). A carbodiimide-mediated reaction between the protein and the bis-hydrazine reagent forms diimide bond derivatives terminating in hydrazide groups (Figure 23.9). (Strept)avidin labeled with adipic acid dihydrazide can form the basis of a carbohydrate detection system using the (strept)avidin–biotin interaction (Bayer *et al*., 1987a, 1990; Bayer and Wilchek, 1990). Glycoconjugates in tissue sections, cells, or blots may be treated with sodium periodate or galactose oxidase to create aldehyde groups on the associated sugar components. Introduction of hydrazide-activated (strept)avidin causes hydrazone bonds to form between the hydrazides and aldehydes, thus specifically targeting glycoproteins and other carbohydrate-containing molecules. Subsequent detection with a biotinylated enzyme allows precise localization of glycoconjugates. Detection in a single step using this strategy is possible using preformed complexes of hydrazide-activated (strept)avidin and a biotinylated enzyme (Figure 23.10).

The activation of (strept)avidin with adipic dihydrazide may be done using the method of Bayer *et al*. (1987a). A summary of this protocol is given below.

Protocol

1. Dissolve 160 mg of adipic acid dihydrazide (Aldrich) in 5 ml of 0.1 M sodium phosphate, pH 6.0. Some heating of the tube under a hot-water tap may be required to help solubilize the compound. Cool to room temperature.
2. Dissolve 50 mg of (strept)avidin in the adipic acid dihydrazide solution.

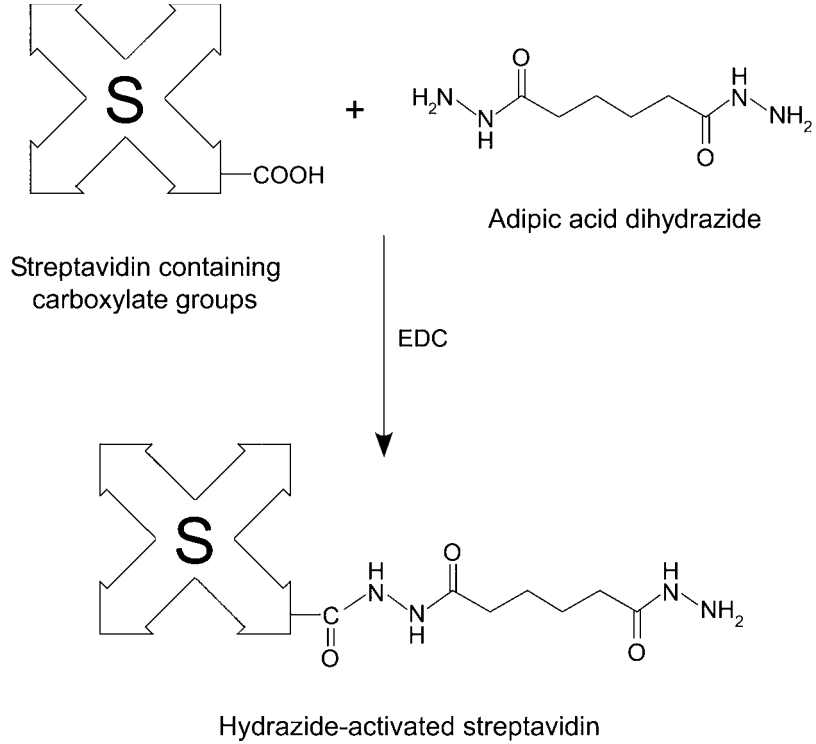


Figure 23.9 Reaction of adipic acid dihydrazide with (strept)avidin produces a hydrazide derivative that is highly reactive toward periodate-oxidized polysaccharides.

3. Add 160 mg of the water-soluble carbodiimide EDC (Thermo Fisher) (Chapter 3, Section 1.1) to the solution, and mix to dissolve.
4. React for 4 hours at room temperature.
5. Dialyze against PBS, pH 7.2 to remove excess reagent and reaction by-products.

Hydrazide-activated (strept)avidin may be stored as a freeze-dried preparation without loss of activity.

6. Biotinylation Techniques

In addition to preparing the (strept)avidin conjugates necessary to develop (strept)avidin–biotin-based systems, the process of modifying targeting molecules with a biotin tag is just as critical and forms the other key component of the interacting complex. Since biotin is a relatively small molecule (MW 244.31), coupling it to macromolecules usually can be done without disturbing the activity or binding capability of either the targeting molecule or the biotin handle. Proteins, carbohydrates, lipid molecules, and nucleic acids can be modified to contain one or more biotins able to strongly interact with (strept)avidin. The technique of biotinylation is made easy

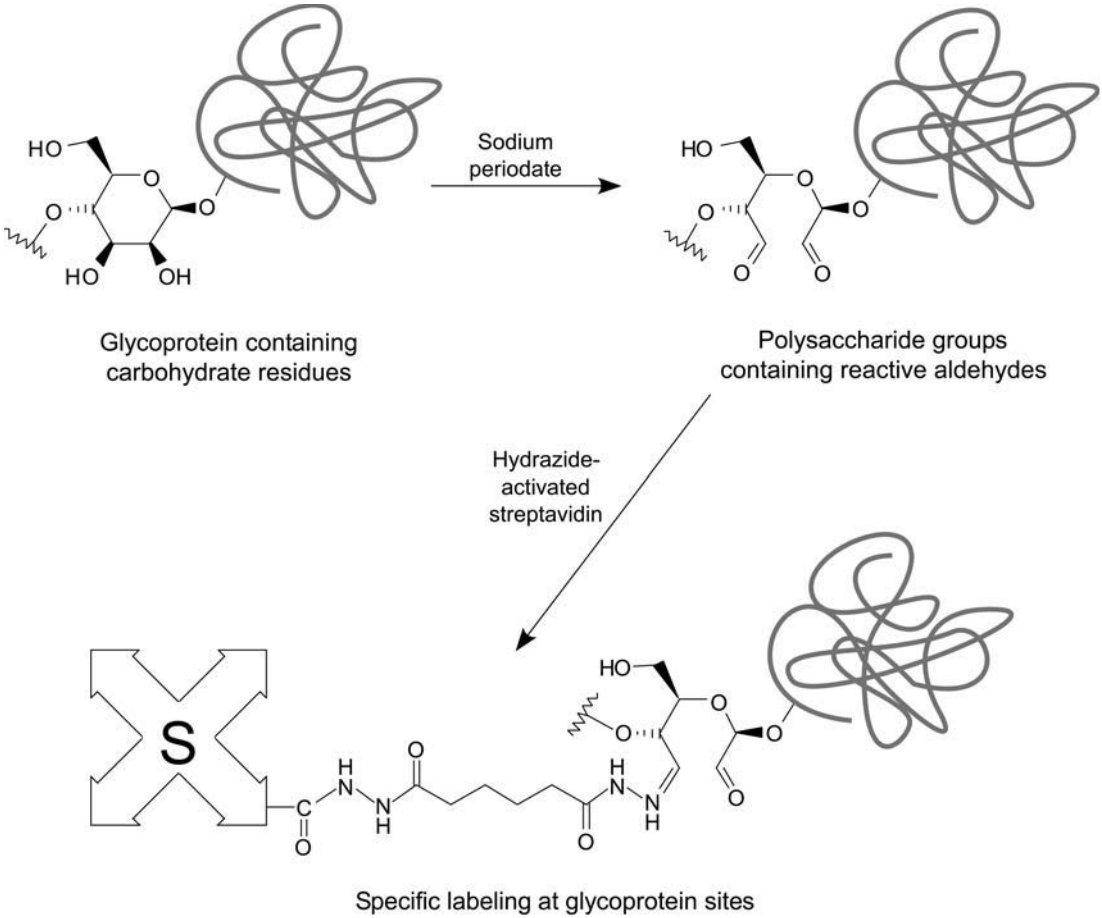


Figure 23.10 Glycoproteins may be oxidized with sodium periodate to generate aldehyde residues. These may be specifically labeled using a hydrazide-streptavidin derivative through hydrazone bond formation. Subsequent detection may be done using biotinylated enzymes.

through the commercial availability of a range of different biotin derivatives having a number of important reactivity and property characteristics useful in (strept)avidin–biotin chemistry.

Chapter 11 and Chapter 18, Section 3, describe the major biotinylation compounds and their properties. Also provided in these sections are suggested protocols for reacting each of these reagents with specific functionalities on macromolecules.

7. Determination of the Level of Biotinylation

It is often important to determine the extent of biotin modification after a biotinylation reaction is complete. Measuring biotin incorporation into macromolecules can aid in optimizing a particular (strept)avidin–biotin assay system. It also can be used to assure reproducibility in

the biotinylation process. The most common method of measuring the degree of biotinylation makes use of the HABA-dye assay (Green, 1965). HABA is 4′-hydroxyazobenzene-2-carboxylic acid. In the absence of biotin, the dye is capable of specifically forming noncovalent complexes with (strept)avidin at its biotin binding sites. Upon binding to (strept)avidin in aqueous solution, HABA exhibits a characteristic absorption band at 500 nm ($\varepsilon = 35{,}500\,M^{-1}cm^{-1}$, expressed as per mole of HABA bound). The addition of biotin to this complex results in displacement of HABA from the binding site, since the affinity constant of the (strept)avidin–biotin interaction ($1.3 \times 10^{15}\,M^{-1}$) is much greater than that for (strept)avidin–HABA ($6 \times 10^{6}\,M^{-1}$). As HABA is displaced, the absorbance of the complex decreases proportionally. Thus, the amount of biotin present in the solution can be determined by plotting the (strept)avidin–HABA absorbance at 500 nm versus the absorbance modulation with increasing concentrations of added biotin. Comparing an unknown biotin-containing sample to this standard response curve can result in the determination of the biotin concentration in the sample.

Since a biotinylated molecule potentially is able to interact with (strept)avidin at its biotin binding sites just as strongly as biotin in solution, the degree of biotinylation may be determined using the HABA method as well. Comparison of the response of a biotinylated protein, for example, with a standard curve of various biotin concentrations allows calculation of the molar ratio of biotin incorporation.

Two variations of the HABA-dye assay for biotinylated proteins are possible. In one approach, the biotinylated protein is digested using the enzyme pronase prior to doing the assay. The digestion process breaks the protein into small fragments, some of which possess biotin modifications. The digestion is done to eliminate any sterically hindered biotinylation sites from not being able to interact with (strept)avidin. The second approach merely uses the intact biotinylated protein in the assay, assuming that the HABA assay results then will provide a truer picture of the level of *accessible* biotin sites on the molecule. Pronase addition obviously is not necessary for assessing biotinylated molecules which are not proteins.

The following protocol describes both of these HABA-based tests for determining the level of biotinylation.

Protocol

1. Dissolve (strept)avidin in 0.05 M sodium phosphate, 0.15 M NaCl, pH 6.0, at a concentration of 0.5 mg/ml. A total of 3 ml of the (strept)avidin solution is required to create a standard curve using known concentrations of biotin and an additional 3 ml is needed for each sample determination.
2. Dissolve the HABA dye (Sigma) in 10 mM NaOH at a concentration of 2.42 mg/ml (10 mM). Prepare about 100 µl of the HABA solution for each 3 ml portion of (strept)avidin solution required.
3. Dissolve the biotinylated protein to be measured in 0.05 M sodium phosphate, 0.15 M NaCl, pH 6.0, at a concentration of 10–20 mg/ml. The amount required is about 100 µl of sample per determination.
4. Dissolve D-biotin in 0.05 M sodium phosphate, 0.15 M NaCl, pH 6.0, at a concentration of 0.5 mM.
5. For the proteolytic digestion procedure, dissolve pronase in water at a concentration of 1 percent (w/v).

6. If pronase digestion of the biotinylated protein is to be done, heat 100 µl of the sample at 56°C for 10 minutes, then add 10 µl of the pronase solution. Allow the sample to digest enzymatically at room temperature overnight. If no pronase digestion is desired, simply use the biotinylated protein solution prepared in step 3 without further treatment.
7. To construct a standard curve of various biotin concentrations, first zero a spectrophotometer at an absorbance setting of 500 nm with sample and reference cuvettes filled with 0.05 M sodium phosphate, 0.15 M NaCl, pH 6.0. Remove the buffer solution from the sample cuvette and add 3 ml of the (strept)avidin solution plus 75 µl of the HABA-dye solution. Mix well and measure the absorbance of the solution at 500 nm. Next add 2 µl aliquots of the biotin solution to this (strept)avidin–HABA solution, mix well after each addition, and measure and record the resultant absorbance change at 500 nm. With each addition of biotin, the absorbance of the (strept)avidin–HABA complex at 500 nm decreases. The absorbance readings are plotted against the amount of biotin added to construct the standard curve.
8. To measure the response of the biotinylated protein sample, add 3 ml of the (strept)avidin solution plus 75 µl of the HABA dye to a cuvette. Mix well and measure the absorbance of the solution at 500 nm. Next, add a small amount of sample to this solution and mix. Record the absorbance at 500 nm. If the change in absorbance due to sample addition was not sufficient to obtain a significant difference from the initial (strept)avidin–HABA solution, add another portion of sample and measure again. Determine the amount of biotin present in the protein sample by using the standard curve. The number of moles of biotin divided by the moles of protein present gives the number of biotin modifications on each protein molecule.

24

Preparation of Colloidal Gold-Labeled Proteins

As early as the first decade of the twentieth century colloidal gold sols containing particles of less than 10 nm were produced by chemical means (Zsigmondy, 1905). However, the application of these inorganic suspensions to protein labeling didn't occur until 1971 when Faulk and Taylor invented the immunogold staining procedure. Since that time, the labeling of targeting molecules, especially proteins, with gold nanoparticles has revolutionized the visualization of cellular or tissue components by electron microscopy (Horisberger *et al*., 1975; Horisberger, 1979). The silver enhancement technique further broadened the application of gold labeling to include light microscopy (Holgate *et al*., 1983). The electron-dense and visually opaque nature of gold labels also provided excellent detection qualities for such techniques as blotting, flow cytometry, cytochemical staining, and hybridization assays (Jackson *et al*., 1990; Gee *et al*., 1991). Double- or triple-labeling systems have been constructed using immunogold methods in tandem with immunoenzymatic techniques to detect more than one antigen at the same time (Gillitzer *et al*., 1990).

This section discusses the properties of gold particles as well as the common methods of labeling proteins and other biomolecules with them. The cited references should be consulted to obtain protocols for using these protein–gold complexes in assay and detection systems.

1. Properties and Use of Gold Conjugates

Colloidal gold suspensions consist of small granules of this transition metal in a stable, uniform dispersion. Viewed under the light or electron microscope, they appear as solid spheres of dense material. In electron microscopy the gold particles are visible as dense, dark markers usually black in appearance. In light microscopy, they can appear as light dots on a darker background due to the high reflectance of the particles or as an orange-red coating where they are localized in large conglomerates on cells or tissues. Colloidal gold particles act as efficient nuclei for deposition of silver, thus markedly enhancing their detection under light microscopy (Danscher and Rytter-Nörgaard, 1983). The same silver–gold combination also provides increased sensitivity in blotting applications (Moeremans *et al*., 1984).

Most preparations of colloidal gold consist of particles varying in diameter from about 5 nm to around 150 nm. The methods of forming small particle gold suspensions of known diameter are discussed in Section 2.

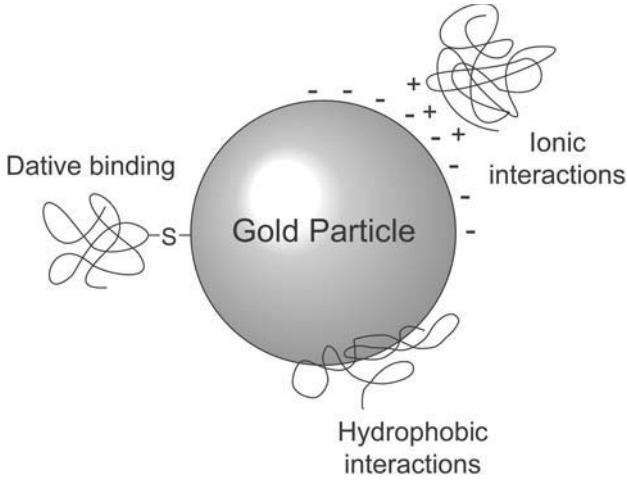


Figure 24.1 Protein binding to gold particles can occur through several types of interactions.

The labeling of macromolecules with gold particles proceeds through a number of rather poorly understood processes. Preparing stable protein–gold complexes depends on several interactions: (a) the electronic attraction between the negatively charged gold particles and the abundant positively charged sites on the protein molecule, (b) an adsorption phenomena involving hydrophobic pockets on the protein binding to the metal surface, and (c) the potential for covalent binding of gold to free sulfhydryl groups, if present (dative binding, see Chapter 2, Section 2.8) (Figure 24.1).

Deryagin and Landau (1941) and Verwey and Overbeek (1948) working independently developed a theory of the behavior of colloidal systems that aids in understanding macromolecular labeling with gold particles. Called the DLVO theory from the initials of the four authors, it views the particles in a sol as consisting of two components producing opposite effects in aqueous suspension. The overlap of the electrical double layer of each particle causes a negative charge on the surface, leading to particle–particle repulsion and stabilizing the sol from aggregation. The other phenomenon is electromagnetic in nature and leads to the potential for Van der Waals attraction between the metal surface and other molecules.

In the colloidal suspension, there exists a balance between the negative charge repulsion and the attractive forces which could cause coagulation. As particles approach each other, an energy barrier must be traversed to overcome the repulsive character of the negative surface and enter the region of Van der Waals attraction. This barrier can be breached by the addition of electrolytes to the solution that can mask the negative charge on each particle. At a certain concentration of electrolytes, the colloid will begin to collapse as the gold particles adsorb onto one another, forming large aggregates and ultimately falling out of suspension.

Electrolyte-mediated coagulation forms the basis for creating all gold conjugates with other molecules. If macromolecules such as proteins are present in the colloidal suspension as the electrolyte concentration is raised to surpass the negative repulsion effects, then adsorption will occur with the protein molecules instead of with other gold particles. Thus, in place of aggregation and collapse of the suspension, labeling occurs.

The most common electrolyte additions in protein–gold labeling are NaCl or buffer salts. If no macromolecules are present, the addition of NaCl would itself cause gold particle coagulation. The aggregation is accompanied by a color change from orange-red to red-violet or blue (Roth and Binder, 1978), and it may be quantified spectrophotometrically by the change in absorbance at 580 nm (Horisberger *et al*., 1975).

In practice, the addition of a protein to a gold sol will result in spontaneous adsorption on the surface of the gold particles due to electrostatic, hydrophobic, and Van der Waals interactions. To prepare labeled proteins, initially the gold suspension is rapidly mixed while a quantity of protein is added. As the gold is bound to the protein molecules, a decrease in the absorbance at 580 nm occurs as the gold particles become stabilized and less coagulated. To check for the completeness of the adsorption process and to determine if the gold particles are totally blocked, a portion of the sol can be removed and an aliquot of NaCl added. If coagulation occurs upon addition of salt (increase in A_{580nm}), more protein should be added to completely stabilize the sol. Finally, many protocols further stabilize the colloidal suspension after protein binding by the addition of polyethylene glycol (PEG) or an immunochemical blocking agent, such as BSA or a solution of dried milk. These blocking agents completely mask any remaining sites of potential gold–gold or gold–protein interactions, thus preventing aggregation or nonspecific binding during assays.

To produce acceptable gold probes, it is often a common practice to add the minimum quantity of protein needed to prevent NaCl-induced aggregation plus about 10–20 percent excess (Horisberger and Rosset, 1977; De Mey *et al*., 1981). Other investigators have reported that the addition of large excesses of protein to the amount of gold present yields conjugates of higher specific activity (Tokuyasu, 1983; Tinglu *et al*., 1984). However, there is some evidence that overloading may cause leaching of loosely bound protein (Horisberger and Clerc, 1985).

As in any conjugation procedure, optimization of the ratios of reactants must be done to obtain the best probes. In labeling proteins with gold particles, several parameters should be considered: (a) the pI of the protein, (b) the pH of the adsorption process, and (c) the quantity of protein charged to the labeling reaction. It is generally believed that most proteins can be made to adsorb maximally at or near their isoelectric point (Norde, 1986). This is the pH of net electrical neutrality for a protein, wherein any electrically induced repulsive or attractive forces are balanced. For many proteins, especially antiserum-derived immunoglobulins, the average pI is a broad band encompassing a range of pH values. Thus, a polyclonal antibody preparation may possess an average pI much different than a particular purified monoclonal.

Geoghegan (1988) determined that as the pH of the adsorption reaction increased beyond the pI range, the percentage of IgG bound to gold particles decreased. However, for high-pI immunoglobulins, coupling at basic pH values increased the coupling yield. Geoghegan also noted that the more immunoglobulin that was charged to the adsorption process, the more ended up being coupled, although the percent bound would decrease.

Thus, while definite standards for the ratio of protein-to-gold are not universally agreed upon, the efficiency of the process can be improved by following these general guidelines: (a) perform the adsorption reaction at a pH within the range of the pI of the protein being modified or at slightly higher pH, (b) charge an amount of protein to the gold particles that is slightly more (by about 10 percent) than necessary to maintain colloidal stability upon addition of NaCl, (c) avoid high overloads of protein, since this may promote subsequent leaching of bound material, (d) evaluate the degree of adsorption and the relative coagulation of the gold particles by measuring the absorbance of the solution at 580 nm, and (e) each protein–gold conjugate should be optimized as to colloidal stability and retention of activity.

An approximation of the correct amount of protein to be added to a gold sol to maintain stability of the colloid can be done using the following protocol (Slot and Geuze, 1984).

Protocol

1. Add 0.25 ml of the gold suspension to separate tubes containing 25 µl of different concentrations of the protein to be adsorbed. The amount of protein required to stabilize 1 ml of most gold sols is in the microgram range. The protein concentrations should be from about 10 µg/100 µl to about 150 µg/100 µl. Mix well.
2. After about 1 minute, add 0.25 ml of 10 percent NaCl to the gold/protein suspension. Mix well.
3. Monitor the stability of the gold sol by its color or by the absorbance of the mixture at 580 nm. As long as the colloid continues to turn blue, and thus forms gold aggregates with addition of electrolyte, the amount of protein added is not sufficient to stabilize the suspension. This condition translates into a decrease in the absorbance at 580 nm. When the concentration of protein added is enough to stabilize the colloidal suspension, the solution no longer changes color (the absorbance at 580 nm no longer decreases).
4. The amount of protein added at the stabilization point plus 10 percent should be used to produce the final protein–gold conjugate.

The use of gold probes in detection systems has a number of advantages. The ability to label macromolecules with a range of gold particle sizes makes it possible to visualize the probe under a variety of microscopic conditions. Gold avoids all the disadvantages of radioactive labels, while being much more stable to quenching or fading than fluorescent probes or enzymatically developed substrate chromophores. A gold-labeled tissue, cell, or blot will maintain its record of staining on a permanent basis. Under sufficient magnification, an assessment of the degree of antigen labeling can be made simply by counting the number of gold particles present per unit area of cell or tissue mass. This cannot be done with other labeling systems, since chemical stains develop an amorphous quality that does not allow differentiation of individual molecules. Finally, gold probes are essentially non-toxic and relatively inexpensive to use.

A variety of biological molecules can be labeled with gold particles. Proteins are perhaps the most common gold probes; toxins, antibodies, immunoglobulin binding proteins such as protein A, enzymes, lectins, avidin and streptavidin, lipoproteins, and glycoproteins all have been labeled with colloidal gold to form highly sensitive reagents. In addition, polymers, hormones, carbohydrates, and lipids have been gold-labeled for various applications. Small hapten molecules co-adsorbed with adjuvant peptides to gold particles make extraordinary immunogen complexes, producing polyclonal antibody responses having very high titers (Pow and Crook, 1993).

Very small gold particles can even be derivatized to contain specific chemical reactive groups for covalent coupling to macromolecules. For instance, an NHS ester-containing gold particle of 1.4 nm is manufactured by Nanoprobes (Stony Brook, NY). Presumably, such derivatives are formed by adsorption of chemically reactive polymers or by dative binding with a sulfhydryl-containing modification reagent.

The following sections discuss the preparation of colloidal gold suspensions of various particle sizes and their use in labeling proteins for detection purposes. Gold-labeled molecules and proteins are available from a number of manufacturers (Janssen, E-Y Labs, and Nanoprobes).

2. Preparation of Mono-Disperse Gold Suspensions for Protein Labeling

Mono-disperse colloidal gold suspensions useful for labeling macromolecules can be produced by a variety of chemical methods. Three main procedures have become common for making particles which fall into predictable particle-size ranges. All of them use reductive processes on chloroauric acid ($HAuCl_4$) to create the spheroidal gold particles. In general, the greater the power and concentration of the reducing agent, the smaller the resultant particles.

To create large-particle colloidal gold dispersions, chloroauric acid normally is treated with sodium citrate. The result is a particle range of about 15–150 nm, depending on the concentration of citrate utilized (Honsberger, 1979; Horisberger and Rosset, 1977; Pow and Morris, 1991). Medium-sized gold particles of diameter between 6 nm and 15 nm (average 12 nm) are formed by treatment with sodium ascorbate as the reductant (although some procedures use trisodium citrate at higher concentrations than the sodium citrate used for making large particles) (Honsberger and Tacchini-Vonlanthen, 1983; Albrechte *et al*., 1989). The smallest gold particles (<5 nm diameter) are created by reduction with either yellow or white phosphorus (Zsigmondy, 1905; Faulk and Taylor, 1971; Horisberger and Rosset, 1977; Pawley and Albrecht, 1988). Particles as small as 2 nm may be created by reduction with sodium borohydride (Bonnard *et al*., 1984).

The following protocols for creating colloidal gold sols are adaptations from the above cited articles. To obtain reproducible preparations, extreme care should be taken in making each batch to maintain the same reagent concentrations, temperatures, and times for the reactions. In each preparation, a color change is noted as the chloroauric acid is reduced from its initial state to the final gold sol. The initial color is typically a brown, purple-red, or dark blue, depending on the reductant used and other conditions. The final color of the mono-disperse colloidal gold preparation is typically red.

2.1. Preparation of 2 nm Gold Particle Sols

1. Prepare 1 ml of a 4 percent $HAuCl_4$ solution in deionized water.
2. Add 375 µl of the chloroauric acid solution plus 500 µl of 0.2 M K_2CO_3 to 100 ml deionized water, cooled on ice to 4°C. Mix well.
3. Dissolve sodium borohydride ($NaBH_4$) in 5 ml of water at a concentration of 0.5 mg/ml. Prepare fresh.
4. Add five 1-ml aliquots of the sodium borohydride solution to the chloroauric acid/carbonate suspension with rapid stirring. A color change from bluish-purple to reddish-orange will be noted as the additions take place.
5. Stir for 5 minutes on ice after the completion of sodium borohydride addition.

2.2. Preparation of 5 nm Gold Particle Sols

1. Prepare 7 ml of a 1 percent $HAuCl_4$ solution in deionized water.
2. Add 6.25 ml of the chloroauric acid solution plus 5.8 ml of 0.1 M K_2CO_3 to 500 ml deionized water. Mix well.
3. In a fume hood, prepare a saturated solution of white phosphorus in diethyl ether, then dilute 1 part of the saturated phosphorus solution with 4 parts of diethyl ether.

4. Add 4.16 ml of the diluted phosphorus solution to the chloroauric acid/carbonate solution with mixing.
5. React at room temperature for 15 minutes.
6. Bring the mixture to a boil and reflux until the color of the suspension turns from brownish to red. This should take no more than about 5 minutes.
7. Cool the sol to room temperature.
8. The pH of the suspension will be around 6. Adjustments to more alkaline conditions for adsorbing macromolecules of higher pI may be done by addition of 0.1 M K_2CO_3 with stirring. Monitor pH of the sol using a gel-filled electrode (Orion Research, No. 9115, Cambridge, MA) (Geoghegan *et al.*, 1980). After pH adjustment, the gold should be used immediately for complexing with a protein or other macromolecule.

2.3. Preparation of 12 nm Gold Particle Sols

1. Prepare 5 ml of a 1 percent $HAuCl_4$ solution in deionized water.
2. Add 4 ml of the chloroauric acid solution plus 4 ml of 0.1 M K_2CO_3 to 100 ml deionized water. Mix well and cool the solution on ice.
3. With rapid mixing of the chloroauric acid/carbonate solution, quickly add 1 ml of a 7 percent sodium ascorbate solution prepared in water. Maintain the solution cooling in an ice bath. Higher temperatures will create larger particle sizes. The color of the solution at this point will turn to a purple-red.
4. Adjust the volume of the reaction to 400 ml with deionized water.
5. Bring the mixture to a boil and reflux until the color of the suspension turns from purple-red to red.
6. Cool the sol to room temperature.
7. The pH of the suspension will be around 6. Adjustments to more alkaline conditions for adsorbing macromolecules of higher pI may be done by addition of 0.1 M K_2CO_3 with stirring. Monitor pH of the sol using a gel-filled electrode (Geoghegan *et al.*, 1980). After pH adjustment, the gold should be used immediately for complexing with a protein or other macromolecule.

2.4. Preparation of 30 nm Gold Particle Sols

1. Prepare 1 ml of a 4 percent $HAuCl_4$ solution in deionized water.
2. Add 0.5 ml of the chloroauric acid solution to 200 ml of deionized water and bring to a boil while mixing.
3. Add to the boiling, rapidly mixing solution of chloroauric acid, 3 ml of a 1 percent sodium citrate solution.
4. Reflux for 30 minutes. The color of the suspension will change from a dark blue to a red as the mono-disperse colloidal gold particles are formed.
5. Cool to room temperature.

Any of the particle sols prepared above may be used to adsorb macromolecules to create gold probes. To concentrate the suspensions, the solutions may be filtered through a small-pore

filter. Centrifugation also may be done. Each protein–gold complexation should be optimized for the proper amount of protein to add to maintain stability of the colloid. This can be done according to the method described in Section 1, this chapter.

3. Preparation of Protein A–Gold Complexes

Protein A–gold probes (as well as other immunoglobulin binding proteins adsorbed to gold) have been used to visualize antibody binding to antigenic sites in tissue sections, cells, and blots (Hearn, 1987; Jemmerson and Agre, 1987; Lethias *et al*., 1987; Yokota, 1988; Bendayan and Garzon, 1988; Bendayan, 1989; Herbener, 1989; Roth *et al*., 1989; Stump *et al*., 1989). Gold labeling of immunoglobulin binding proteins provides "universal" probes for detection of any antibody–antigen interaction (Figure 24.2). Thus, only one gold-labeled reagent need be prepared to visualize many different immunochemical targeting procedures. This avoids having to make antibody–gold probes for each specific immunoglobulin used. The downside of this approach, however, is the potential nonspecificity of protein A in binding other antibodies that may be present within the sample.

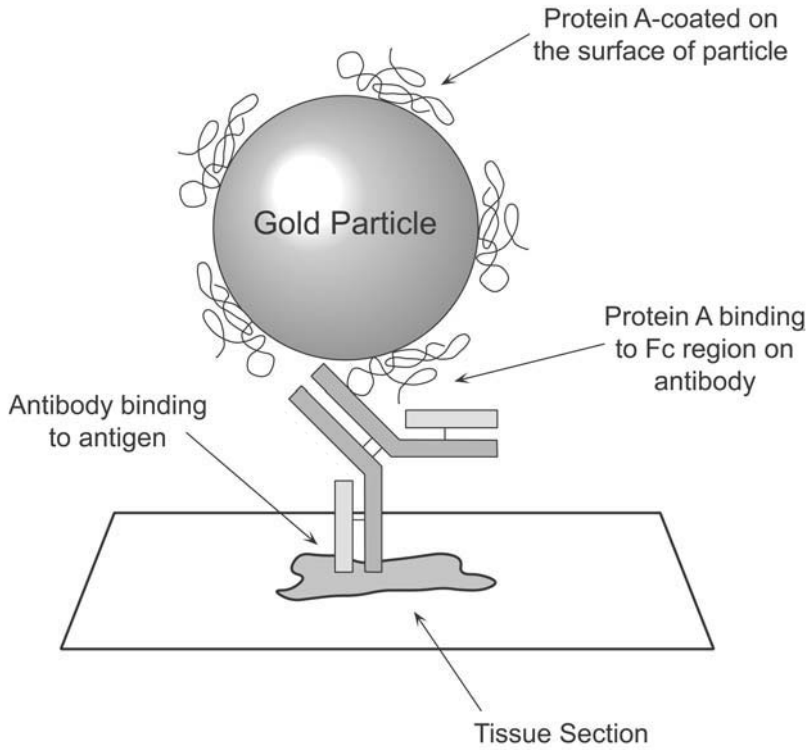


Figure 24.2 Antigens may be detected in cells or tissue sections through the use of protein A-coated gold particles. The binding of a specific primary antibody to its target antigen can be localized by the immunoglobulin binding capability of protein A, which occurs in the Fc region of the antibody.

Protocol

1. Determine the minimum amount of protein A required to stabilize the colloidal gold sol being used. The colloidal suspension should be adjusted, if needed, with 0.1 M K_2CO_3 to pH 6–7. Measure the pH of the sol using a gel-filled electrode. Determining the stabilization amount of protein A can be done according to the method described in Section 1, this chapter.
2. Mix a stabilizing amount of protein A plus an additional 10 percent with the appropriate volume of colloidal gold. For example, Herbener (1989) mixed 10 ml of a 14 nm gold particle sol at pH 6.9 with 0.3 mg of protein A dissolved in 0.2 ml water. Mix well.
3. After 1 minute, add 250 µl of 1 percent polyethylene glycol (MW 20,000) per 10 ml of gold sol used. The PEG helps to stabilize further the sol against aggregation.
4. Stir for an additional 5 minutes.
5. To remove excess protein A, centrifuge the preparation at a minimum of 50,000 g for 30 minutes to several hours (4°C), depending on the size of the particles and the amount of solution. Discard the supernatant, and resuspend the protein A–gold pellet in 0.01 M sodium phosphate, pH 7.4, containing 1 percent PEG.

4. Preparation of Antibody–Gold Complexes

Immunocytochemical staining with antibody–gold probes is a powerful way to detect, localize, and quantify antigen molecules in tissue sections and cells (Figure 24.3). Metabolic processes can be followed, epitope mapping of the structural characteristics of macromolecules can be

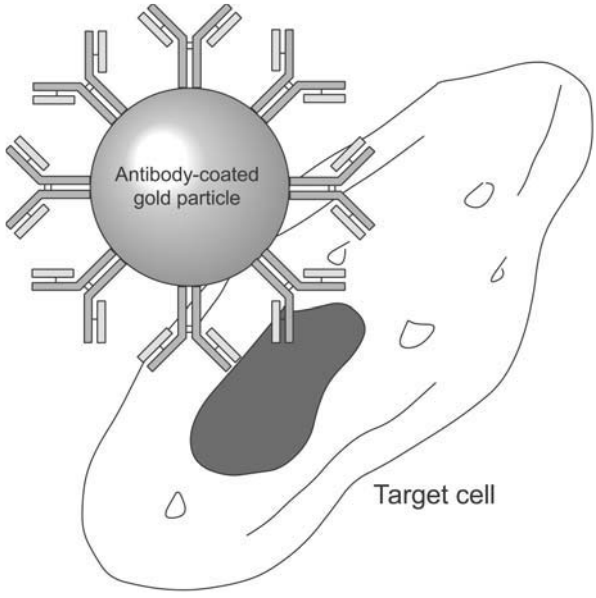


Figure 24.3 Antibodies coated on colloidal gold particles can be used to detect specific antigens in cells.

done, and detection of pathogens or other foreign substances within cells can be accomplished using gold-labeled antibodies (Ellis *et al.*, 1988; Albrecht *et al.*, 1989; Cramer *et al.*, 1989; De Waele *et al.*, 1989; Nielsen *et al.*, 1989; Martinez-Ramon *et al.*, 1990; van den Brink *et al.*, 1990).

The optimal coupling pH for an antibody should be determined by measurement of the relative pI range of the immunoglobulin. Many antibodies, however, adsorb best at a pH of 8–9. The optimal level of protein addition to the gold sol to prevent aggregation should be determined according to the method of Section 1, this chapter. In addition, bovine serum albumin (BSA) is often added instead of PEG (see the protein A coupling procedure, described previously) to further stabilize the antibody–gold suspension.

Protocol

1. Determine the minimum amount of antibody required to stabilize the colloidal gold sol being used. The colloidal suspension should be adjusted, if needed, with 0.1 M K_2CO_3 or NaOH to pH 8–9. Measure the pH of the sol using a gel-filled electrode. Determining the stabilization amount of antibody can be done according to the method described in Section 1, this chapter.
2. Mix a stabilizing amount of antibody plus an additional 10 percent with the appropriate volume of colloidal gold. For example, Geoghegan (1988) found that an addition of 10–14 μg of antibody per ml of gold colloid resulted in stable preparations. Mix well after addition of antibody to the gold suspension.
3. After 1 minute, add a quantity of 10 percent BSA to bring the concentration to 0.25 percent in the antibody–gold suspension. The BSA helps to stabilize further the sol against aggregation and also blocks nonspecific binding sites. Alternatively, PEG may be added according to step 3 of Section 3, this chapter.
4. Stir for an additional 5 minutes.
5. To remove excess IgG, centrifuge the preparation at a minimum of 50,000 g for 30 minutes to several hours (4°C), depending on the size of the particles and the amount of solution. Discard the supernatant, and resuspend the antibody–gold pellet in 0.01 M sodium phosphate, pH 7.4, containing 0.25 percent BSA (or 1 percent PEG, as desired).

5. Preparation of Lectin–Gold Complexes

Lectins, or proteins with specific binding sites for carbohydrates, can be used as targeting molecules to localize particular glycoconjugates such as glycoproteins or glycolipids on cell surfaces (Figure 24.4). Labeled with gold particles, lectins are important probes for detection of cell-surface components, intracellular receptors, and in immunological or biochemical assay procedures (Bog-Hansen *et al.*, 1978; Kimura *et al.*, 1979; Nicolson, 1978; Roth, 1983; Benhamou *et al.*, 1988; Nakajima *et al.*, 1988).

The following generalized protocol is an adaptation for the labeling of 15 nm gold particles with *Aplysia* gonad lectin, as described by Benhamou *et al.*, 1988. Each lectin–gold preparation will have its own unique pH optimum and ratio of lectin-to-gold for the absorption process.

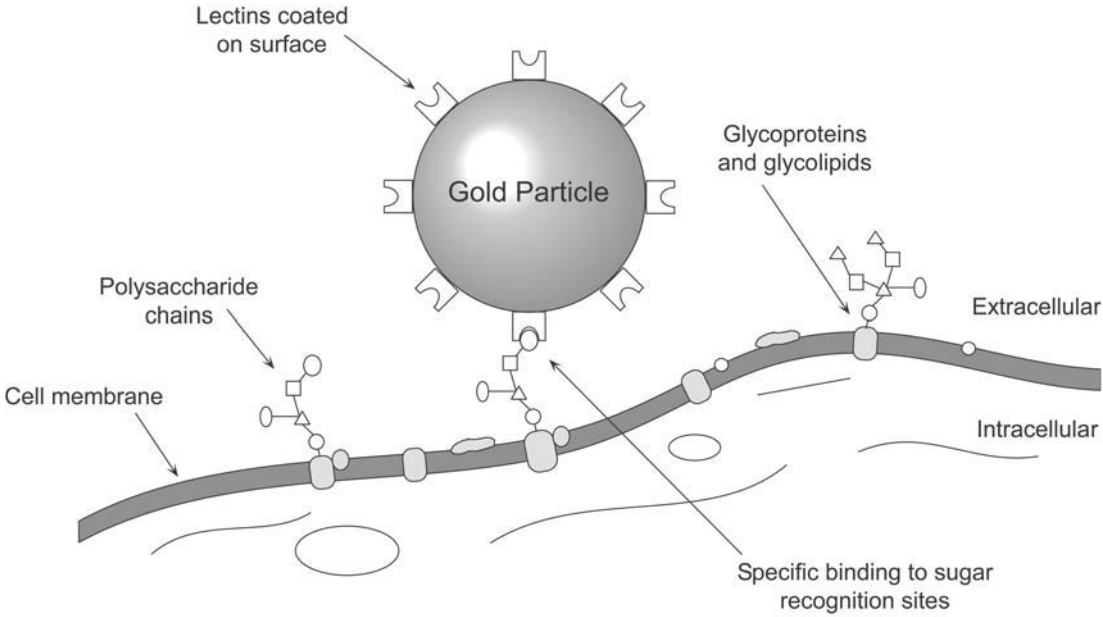


Figure 24.4 Lectins coated on gold particles can be used to detect specific carbohydrate sequences in cell-surface glycoconjugates.

Protocol

1. Determine the minimum amount of lectin required to stabilize the colloidal gold sol being used. The colloidal suspension should be adjusted, if needed, with 0.1 M K_2CO_3 or NaOH to a pH equal to or slightly above the pI of the lectin being used. For *Aplysia* gonad lectin, the optimal pH for adsorption was determined to be 9.5. Nakajima *et al.* (1988) include pI conditions for a number of different lectins. Measure the pH of the sol using a gel-filled electrode. Determining the stabilization amount of lectin can be done according to the method described in Section 1, this chapter.
2. Mix a stabilizing amount of lectin plus an additional 10 percent with the appropriate volume of colloidal gold. For example, Benhamou *et al.*, 1988, found that an addition of 5 μg of lectin per ml of gold colloid resulted in stable preparations. However, in their final lectin–gold conjugate preparation a 5-fold increase in this ratio (25 μg lectin/ml gold) was used to stabilize fully the sol. Mix well after addition of lectin to the gold suspension.
3. After 1 minute, add 250 μl of 1 percent polyethylene glycol (MW 20,000) per 10 ml of gold sol used. The PEG helps to stabilize further the sol against aggregation.
4. Stir for an additional 5 minutes.
5. To remove excess lectin (particularly important if the 5-fold excess ratio is used), centrifuge the preparation at a minimum of 50,000 g for 30 minutes to several hours (4°C), depending on the size of the particles and the amount of solution. Discard the supernatant, and resuspend the lectin–gold pellet in 0.01 M sodium phosphate, pH 7.4, containing 1 percent PEG.

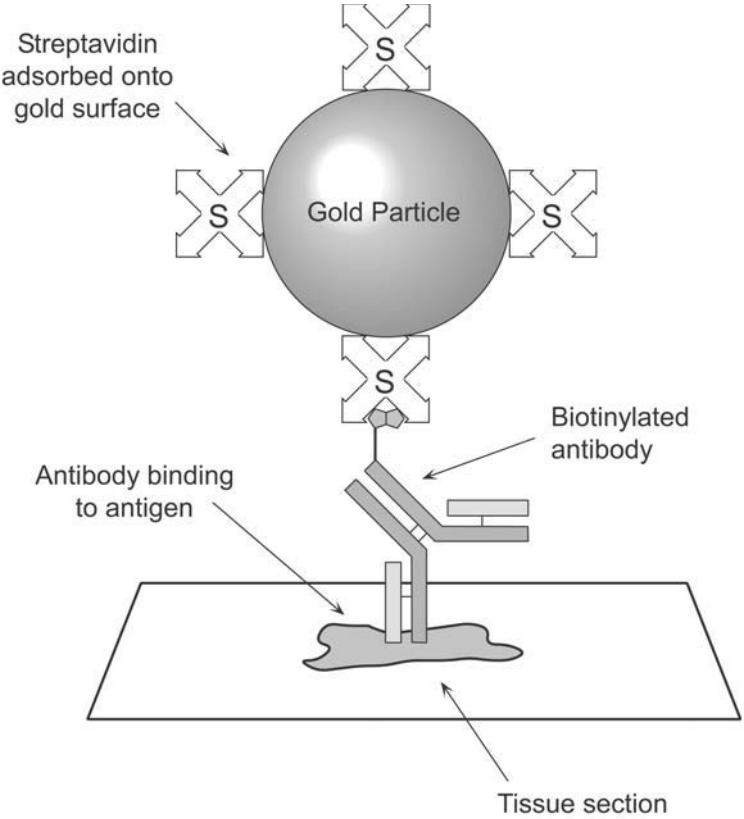


Figure 24.5 Streptavidin-coated gold particles can be used to detect biotinylated antibodies that are bound to specific antigenic determinants.

6. Preparation of (Strept)avidin–Gold Complexes

Avidin– or streptavidin–gold conjugates can be used to detect, localize, or quantify the binding of biotinylated molecules in cells, tissue sections, or blots (Morris and Saelinger, 1984; Bonnard *et al*., 1984; Gillitzer *et al*., 1990; Bronckers *et al*., 1987) (Figure 24.5). These reagents are similar to the use of protein A–gold complexes in detecting immunoglobulins (Section 3, this chapter) in that they are "universal" for detecting any biotin-labeled molecules. Thus, targeting molecules need not be directly modified with gold, only biotinylated so that they are able to interact with avidin– or streptavidin–gold conjugates. See Chapter 11 and Chapter 18, Section 3 for biotinylation reagents and protocols that can be used to add a biotin tag to macromolecules. Also, see Chapter 23 for additional information on (strept)avidin–biotin techniques, including conjugation protocols.

The following protocol is based on the method of Morris and Saelinger (1984) for the labeling of succinylated avidin with gold particles of 5.2 nm diameter. Succinylated avidin was used to reduce the pI of the protein, thus eliminating nonspecific binding due to the strong positive

charge of the native tetramer. Alternatively, streptavidin or NeutrAvidin (Thermo Fisher) can be used to coat gold particles without the need for succinylation.

Protocol

1. Prepare a 200 ml gold sol by using white phosphorus reduction as described in Section 2, this chapter.
2. Prepare 5 ml of a 1 mg/ml succinylated avidin solution by dissolving the protein in 50 mM sodium phosphate, pH 7.5.
3. With stirring, add the succinylated avidin solution to the colloidal gold suspension at room temperature.
4. React for 30 minutes with constant mixing.
5. Remove excess protein by centrifugation at a minimum of 50,000 g for several hours.
6. Suspend the succinylated avidin–gold pellet in 50 mM Tris, 0.15 M NaCl, pH 7.5, containing 0.5 mg/ml PEG (MW 20,000).
7. Centrifuge again under the same conditions to assure complete removal of non-adsorbed protein.
8. Resuspend the pellet in Tris buffer containing PEG and store at 4°C.

A similar protocol has been used by Bonnard *et al.* (1984) in the preparation of streptavidin–gold probes.

Protocol

1. Prepare 20 ml of a gold sol by the white phosphorus method described in Section 2, this chapter.
2. Dissolve streptavidin in 0.1 M sodium phosphate buffer, pH 7.4, at a concentration of 1 mg/ml.
3. With stirring, add the streptavidin solution to the gold suspension. Immediately add 200 µl of 1 M sodium bicarbonate.
4. React for 10 minutes at room temperature.
5. To stabilize further the colloid, add 200 µl of 2 percent PEG 6000.
6. Centrifuge the streptavidin–gold suspension at a minimum of 50,000 g for several hours to remove excess protein solution.
7. Resuspend the pellet in 0.1 M sodium phosphate, 0.02 percent PEG, pH 7.4, and store at 4°C.

25

Modification with Synthetic Polymers

Modification or attachment of proteins or other molecules with synthetic polymers can provide many benefits for both *in vivo* and *in vitro* applications. Covalent coupling of polymers to large macromolecules can alter their surface and solubility properties, creating increased water solubility or even organic solvent solubility for molecules normally sparingly miscible in such environments. Polymer modification of foreign molecules can provide increased biocompatibility, reducing the immune response, increasing *in vivo* stability, and delaying clearance by the reticuloendothelial system. Modification of enzymes with polymers can dramatically enhance their stability in solution. Polymer attachment can provide cryoprotection for proteins sensitive to freezing. Polymers with multivalent reactive sites can be used to couple numerous small molecules for creating pharmacologically active agents that possess long half-lives in biological systems. Similar complexes can be formed to create highly potent immunogens consisting of hapten–polymer conjugates for induction of an antibody response toward the hapten. Polymer modification of surfaces can effectively mask the intrinsic character of the surface and thus prevent nonspecific protein adsorption. Finally, multifunctional polymers can serve as extended crosslinking agents for the conjugation of more than one molecule of one protein to multiple numbers of a second molecule, creating large complexes with increased sensitivity or activity in detecting or acting upon target analytes.

Many polymers have been studied for their usefulness in producing pharmacologically active complexes with proteins or drugs. Synthetic and natural polymers such as polysaccharides, poly(L-lysine) and other poly(amino acids), poly(vinyl alcohols), polyvinylpyrrolidinones, poly(acrylic acid) derivatives, various polyurethanes, and polyphosphazenes have been coupled to with a diversity of substances to explore their properties (Duncan and Kopecek, 1984; Braatz *et al.*, 1993). Copolymer preparations of two monomers also have been tried (Nathan *et al.*, 1993).

The two polymers most often used in these applications are dextran and polyethylene glycol (PEG). Both polymers consist of repeating units of a single monomer—glucose in the case of dextran and an ethylene oxide basic unit in the case of PEG. The polymers may be composed of linear strands or branched constructs. An additional similarity is that both of them possess hydroxyl and ether linkages, lending hydrophilicity and water-solubility to the molecules. Dextran and PEG can be activated through their hydroxyl groups by a number of chemical

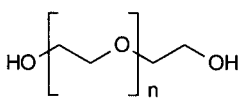
methods to allow efficient coupling of other molecules. Dextran can be activated at multiple sites throughout its chain, since each monomer contains hydroxyl resides. PEG, by contrast, only has hydroxyls at the termini of each polymer strand. Derivatives of both polymers are commercially available.

The following sections discuss the major properties and conjugation chemistries associated with the use of these polymers in modifying or conjugating proteins and other molecules.

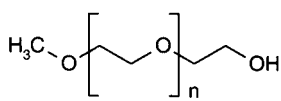
1. Protein Modification with Activated Polyethylene Glycols

Since the first report by Abuchowski *et al.* (1977a, b) concerning the alteration of immunological properties toward bovine serum albumin (BSA) that had been modified with PEG, the interest in polymer modification of biological molecules has grown incessantly. PEG coupled to other molecules can be used for altering solubility characteristics in aqueous or organic solvents (Inada *et al.*, 1986), for modulation of the immune response (Delgado *et al.*, 1992), to increase the stability of proteins in solution (Berger and Pizzo, 1988), to enhance the half-life of substances *in vivo* (Knauf *et al.*, 1988), to aid in penetrating cell membranes, to alter pharmacological properties (Dunn and Ottenbrite, 1991), to increase biocompatibility, especially toward implanted foreign substances, and to prevent protein adsorption to surfaces.

PEG consists of repeating units of ethylene oxide which terminate in hydroxyl groups on either end of a linear chain. Some constructs have branches that have multiple linear strands emanating from these points. PEG is made from the anionic polymerization of ethylene oxide, resulting in the formation of polymer strands of various potential molecular weights, depending on the polymerization conditions. Thus, all polymeric PEGs are polydisperse and exist as distribution of multiple lengths and molecular weights. Most forms of PEG useful in bioconjugate applications have molecular weights less than 20,000 and are soluble both in aqueous solution and in many organic solvents.



PEG
Poly(ethylene glycol)



mPEG
Monomethoxy-
Poly(ethylene glycol)

Unlike the PEG molecules formed from anionic polymerization techniques, there now exist highly discrete forms of the polymer made by controlled addition of small PEG units to create chains of exacting molecular size. These discrete PEGs have a single molecular weight and do not display the polydispersity of the traditional PEG polymers. See Chapter 18 for a complete discussion of discrete PEG-based reagents and their applications.

Since the polymer backbone of PEG is not of biological origin, it is not readily degraded by mammalian enzymes (although some bacterial enzymes will break it down). This property results in only slow degradation of the polymer when used *in vivo*, thus extending the half-life of modified substances. PEG modification serves to mask any molecule that it is coupled to—the "PEGylated" molecule being protected from immediate breakdown or from being complexed and inactivated by immunoglobulins in the bloodstream.

PEG's properties in solution are especially unusual, frequently displaying amphiphilic tendencies, having the ability to both solubilize in aqueous layers and in hydrophobic membranes or organic phases. The partitioning quality of PEG across membranes is important in aiding the formation of hybridomas in the production monoclonal antibodies (Goding, 1986b). The partitioning characteristics of PEG also create the ability to use it in aqueous two-phase systems for the purification of biological molecules (Johansson, 1992).

PEG in solution is a highly mobile molecule that creates a large exclusion volume for its molecular weight, much larger in fact than proteins of comparable size. Whether in solution or attached to other insoluble supports or surfaces, PEG has a tendency to exclude other polymers. This property forms a protein-rejecting region that is effective in preventing nonspecific protein binding (Bergstrom *et al*., 1992). Conjugation with PEG can create the same exclusion effects surrounding a macromolecule, even preventing interaction between a ligand and its target (Klibanov *et al*., 1991), an enzyme and its substrate (Berger and Pizzo, 1988), or the immune system and a foreign substance (Davis *et al*., 1979). Thus, PEG-modified molecules display low immunogenicity, have good resistance to proteolytic digestion, and survive in the bloodstream for extended periods (Abuchowski *et al*., 1977a; Dreborg and Akerblom, 1990).

PEG can be conjugated to other molecules through its two hydroxyl groups at the ends of each linear chain. This process is typically done by the creation of a reactive electrophilic intermediate that is capable of spontaneously coupling to nucleophilic residues on a second molecule. To prevent the potential for crosslinking when using a bifunctional polymer, monofunctional PEG polymers can be used which contain one end of each chain blocked with a methyl ether group. Monomethoxypolyethylene glycol (mPEG) contains only one hydroxyl group per linear chain, thus limiting activation and coupling to one site and preventing the crosslinking and polymerization of modified molecules. The mPEG derivative also stabilizes the blocked end to degradation in solution.

1.1. Trichloro-*s*-triazine Activation and Coupling

The most common activation methods for PEG create amine-reactive derivatives that can form amide or secondary amine linkages with proteins and other amine-containing molecules. The oldest method of PEG activation is through the use of trichloro-s-triazine (TsT; cyanuric chloride) (Abuchowski *et al*., 1977). TsT is a symmetrical heterocyclic compound containing three reactive acyl-like chlorines. This reagent and its derivatives are extensively used in industrial applications to form strong covalent bonds between dye molecules and fabrics. The compound also has been used to activate affinity chromatography supports for the coupling of amine-containing ligands (Finlay *et al*., 1978). Reaction of the TsT with PEG results in the formation of an activated derivative with an ether bond to the hydroxyl group of the polymer. If mPEG is used, TsT activation will be restricted to the one free hydroxyl, thus forming a monovalent intermediate that can be coupled to proteins without polymerization (Figure 25.1).

The three reactive chlorines on TsT have dramatically different reactivities toward nucleophiles in aqueous solution. The first chlorine is reactive toward hydroxyls as well as primary and secondary amine groups at 4°C and a pH of 9.0 (Abuchowski, 1977a; Mumtaz and Bachhawat, 1991). Once the first chlorine is coupled, the second one requires at least room temperature conditions at the same pH to react efficiently. If two chlorines are conjugated to nucleophilic groups, the third is even more difficult to couple, requiring at least 80°C at alkaline

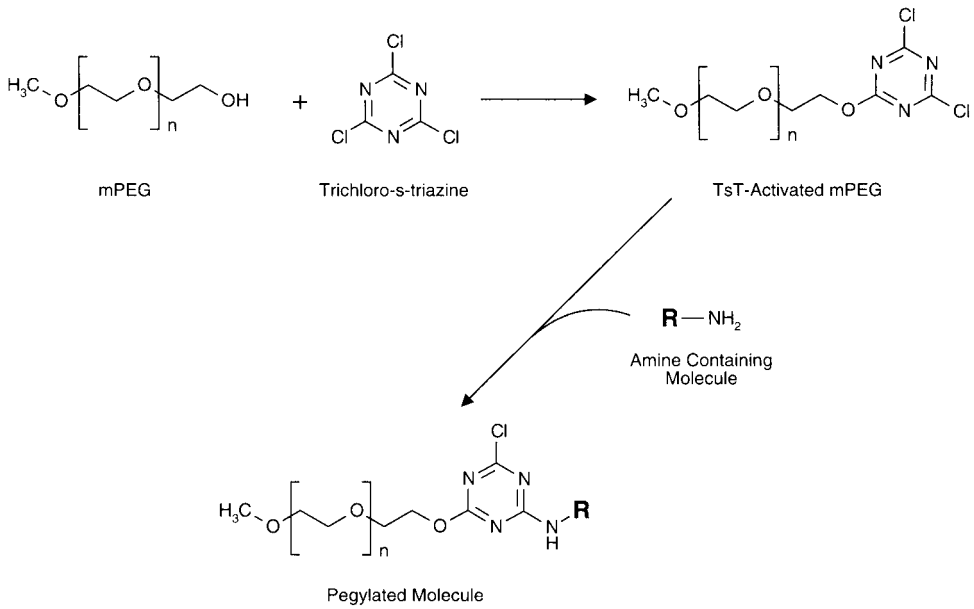


Figure 25.1 mPEG polymers may be activated by trichloro-*s*-triazine for the modification of amine-containing molecules.

pH. After activation of mPEG with TsT, it is therefore, for all practical purposes, only possible to couple one additional component to the triazine ring.

TsT activation provides a simple route to an amine-reactive PEG derivative and it has been used extensively as an activation method for modifying proteins (Wieder *et al.*, 1979; Zalipsky and Lee, 1992; Gotoh *et al.*, 1993). The modification of primary amine-containing molecules such as proteins is pH dependent. At physiological pH values, the reaction will proceed slower than in a more alkaline pH environment. Optimal derivatization efficiency is reached at conditions equal to or above pH 9.0. However, TsT reactivity is not exclusive toward amines. TsT–mPEG modification of proteins can result in modifying other nucleophilic groups such as sulfhydryls and the phenolate ring of tyrosine. In addition, there is potential for toxicity associated with TsT and its derivatives—an especially important consideration for *in vivo* use.

The following protocol for mPEG activation using TsT and its coupling to proteins is based on the protocols of Abuchowski *et al.* (1977b) and Gotoh *et al.* (1993).

Protocol for the Activation of mPEG with TsT

Note: All operations should be done in a fume hood. Dispose of hazardous waste according to EPA guidelines.

1. Dissolve 5.5 g of TsT in 400 ml of anhydrous benzene which contains 10 g of anhydrous sodium carbonate.
2. Add to the TsT solution, 50 g of mPEG-5000 (monomethoxypolyethylene glycol having a molecular weight of 5000). Mix well to dissolve.

3. React overnight at room temperature with stirring.
4. Filter the solution through a glass-fiber filter pad and slowly add, with stirring, 600 ml of petroleum ether (bp 35–60°C).
5. Collect the precipitated product by filtration and redissolve it in 400 ml of benzene. Repeat steps 4–5 several times to assure complete removal of unreacted TsT. The residual TsT may be detected by HPLC using a 250 × 3.2 mm LiChrosorb (5 μm particle size) column from E. Merck. The separation is done using a mobile phase of hexane, and peaks are detected with a UV detector.
6. Remove excess solvents by rotary evaporation. The TsT–mPEG should be used immediately or stored in anhydrous conditions at 4°C.

Protocol for Coupling of TsT–mPEG to Proteins

1. Dissolve the protein to be modified with TsT–mPEG in ice-cold 0.1 sodium borate, pH 9.4, at a concentration of 2–10 mg/ml. Other buffers at lower pH values (down to pH 7.2) can be used and still obtain modification, but the yield will be less. Avoid amine-containing buffers such as Tris or the presence of sulfhydryl-containing compounds, such as disulfide reductants.
2. Slowly add TsT–mPEG to the protein solution at a level of at least a 5-fold molar excess over the desired modification level. For example, Gotoh *et al.* (1993) added 100 lmg of TsT–mPEG-5000 to 19 mg of protein dissolved in 6 ml of buffer. Add the polymer over a period of about 15 minutes with stirring at 4°C.
3. React for 1 hour at 4°C.
4. Remove excess TsT–mPEG by dialysis or gel filtration using a column of Sephacryl S-300.

1.2. NHS Ester and NHS Carbonate Activation and Coupling

Carboxylate groups activated with N-hydroxysuccinimide (NHS) esters are highly reactive toward amine nucleophiles. In the mid-1970s, NHS esters were introduced as reactive ends of crosslinking reagents (Bragg and Hou, 1975; Lomant and Fairbanks, 1976). Their excellent reactivity at physiological pH quickly established NHS esters as the major amine-coupling chemistry in bioconjugate chemistry.

NHS ester-containing compounds react with nucleophiles to release the NHS leaving group and form an acylated product (Chapter 2, Section 1.4). The reaction of such esters with sulfhydryl or hydroxyl groups is possible, but doesn't yield stable conjugates, forming thioesters or ester linkages. Both of these bonds typically hydrolyze in aqueous environments or can undergo transesterification reactions. Histidine side-chain nitrogens of the imidazolyl ring also may be acylated with an NHS ester reagent, but they too hydrolyze rapidly (Cuatrecasas and Parikh, 1972). Reaction with primary and secondary amines, however, creates stable amide and imide linkages, respectively, that don't readily break down. In protein molecules, NHS ester groups primarily react with the α-amines at the N-terminals and the ε-amines of lysine side chains, due to their relative abundance.

PEG contains no carboxylate groups in its native state, but can be modified to possess them by reaction with anhydride compounds. Either PEG or mPEG may be acylated with anhydrides

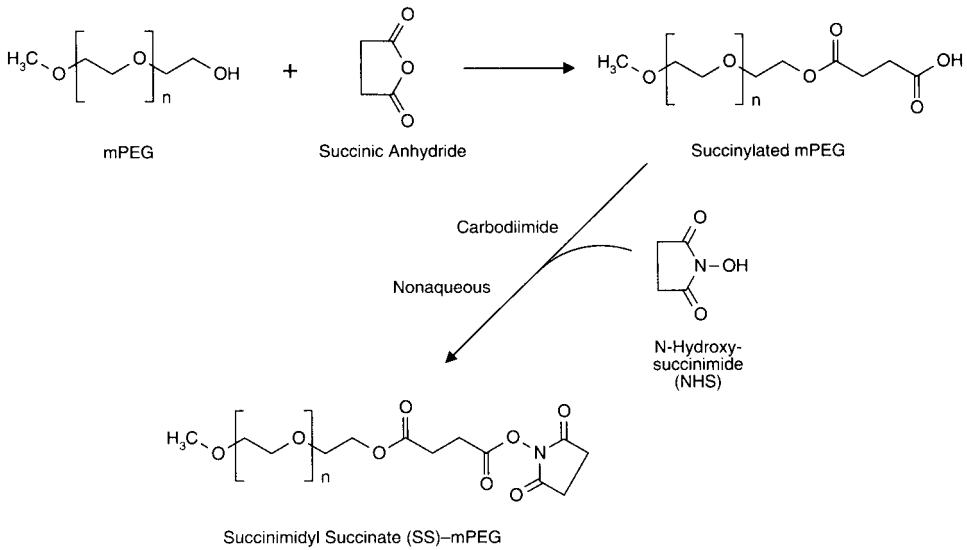


Figure 25.2 mPEG may be derivatized with succinic anhydride to produce a carboxylate end. A reactive NHS ester can be formed from this derivative by use of a carbodiimide-mediated reaction under nonaqueous conditions. The succinimidyl succinate-mPEG is highly reactive toward amine nucleophiles.

to yield ester derivatives terminating in free carboxylate groups. Modification of PEG with succinic anhydride or glutaric anhydride gives *bis*-modified products having carboxylates at both ends. Modification of mPEG yields the mono-substituted derivative containing a single carboxylate. Creation of the succinimidyl succinate and succinimidyl glutarate derivative of PEG was described by Abuchowski *et al.* (1984). A method for the succinylation of mPEG can be found in Section 1.3, this chapter. Subsequent formation of the NHS ester derivatives of these acylated PEG compounds produce highly reactive polymers that can be used to modify amine-containing molecules under mild conditions and with excellent yields (Figures 25.2 and 25.3). The main deficiency of the succinimidyl succinate or succinimidyl glutarate activation procedures is the potential for hydrolysis of the ester bond formed by acylation of the hydroxyl end groups of mPEG.

A modification of the anhydride-acylation route to obtaining reactive NHS ester–PEG compounds was introduced by Zalipsky *et al.* (1991, 1992). In this approach, the terminal hydroxyl group of mPEG is treated with phosgene to give a reactive intermediate, an mPEG-chloroformate compound. Next, the addition of *N*-hydroxysuccinimide (NHS) gives the succinimidyl carbonate (SC) derivative (Figure 25.4). Nucleophiles, such as the primary amino groups of proteins, can react with the SC to give stable carbamate (aliphatic urethane) bonds (Figure 25.5). The linkage is identical to that obtained through *N*,*N*′-carbonyldiimidazole (CDI) activation of hydroxyl groups with subsequent coupling of amines (Chapter 3, Section 3, and this chapter, Section 1.4). However, the reactivity of the SC is much greater than that of the imidazole carbamate formed as the active species in CDI activation.

Unlike the succinimidyl succinate or succinimidyl glutarate activation methods, SC chemistry does not suffer from the presence of a labile ester bond. The intermediate reactive NHS carbonate may hydrolyze in aqueous solution to release NHS and CO_2, essentially regenerating

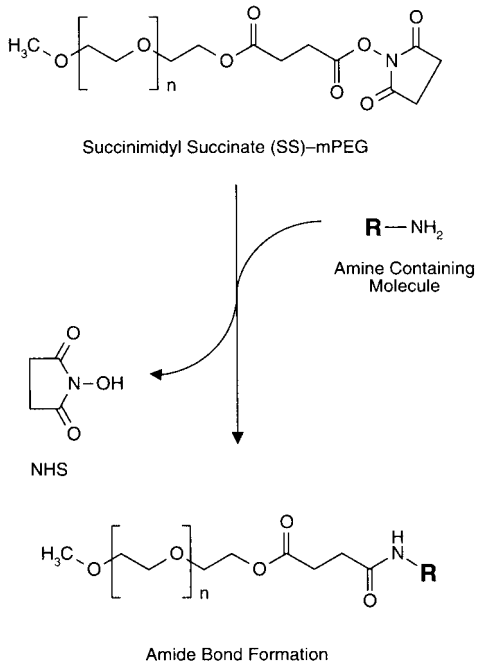


Figure. 25.3 Succinimidyl succinate–mPEG may be used to modify amine-containing molecules to form amide bond derivatives. The ester bond of the succinylated mPEG, however, is subject to hydrolysis.

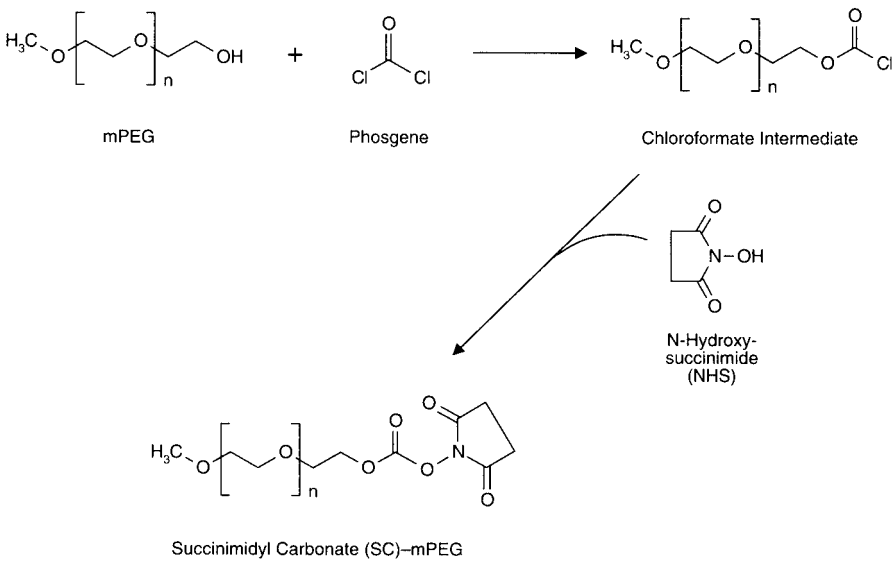


Figure 25.4 An SC derivative of mPEG was first prepared through the use of phosgene to form a chloroformate intermediate. Reaction with NHS gives the amine-reactive SC–mPEG.

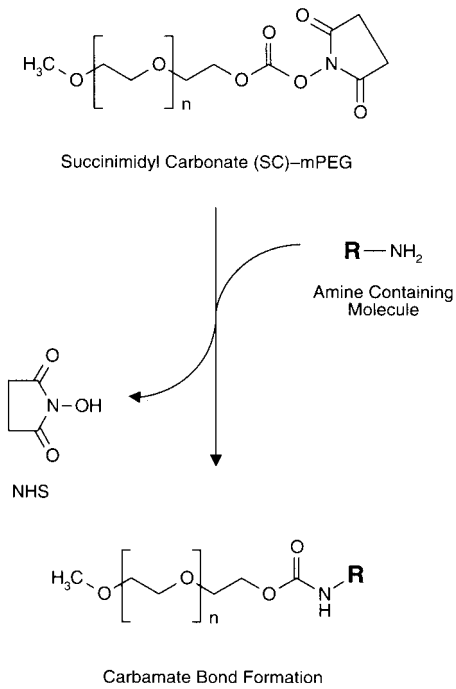


Figure 25.5 An SC–mPEG can be used to modify amine-containing molecules to form stable carbamate linkages.

the underivatized PEG hydroxyl. After coupling to amine-containing molecules, however, the resultant carbamate linkage stabilizes the chemistry to the point that a modified molecule will not lose PEG by hydrolytic cleavage. For these reasons, the SC method of PEG activation and coupling has become the chemistry of choice for attaching the polymer to amine-containing proteins and other molecules.

A modification of Zalipsky's method by Miron and Wilchek (1993) simplifies the creation of the SC-activated species. Instead of using highly toxic phosgene to form a chloroformate intermediate and then reacting with NHS, the new procedure utilizes either *N*-hydroxysuccinimidyl chloroformate or *N*,*N*′-disuccinimidyl carbonate (DSC; Chapter 4, Section 1.7) to produce the SC–PEG in one step (Figure 25.6). Since both activation reagents are commercially available, creating an amine-reactive PEG derivative has never been easier.

The following procedure is based on the Miron and Wilchek modification of Zalipsky's method.

Protocol for the Activation of PEG with *N*-Succinimidyl Chloroformate or *N*,*N*′-Disuccinimidyl Carbonate

Caution: The steps using flammable solvents, especially diethyl ether, should be done in a fume hood.

1. Dissolve 5 g PEG or mPEG (MW 5,000; 1 lmmol) in 25 ml of dry dioxane. Heating in a water bath may be necessary to solubilize fully the polymer. Cool to room temperature.

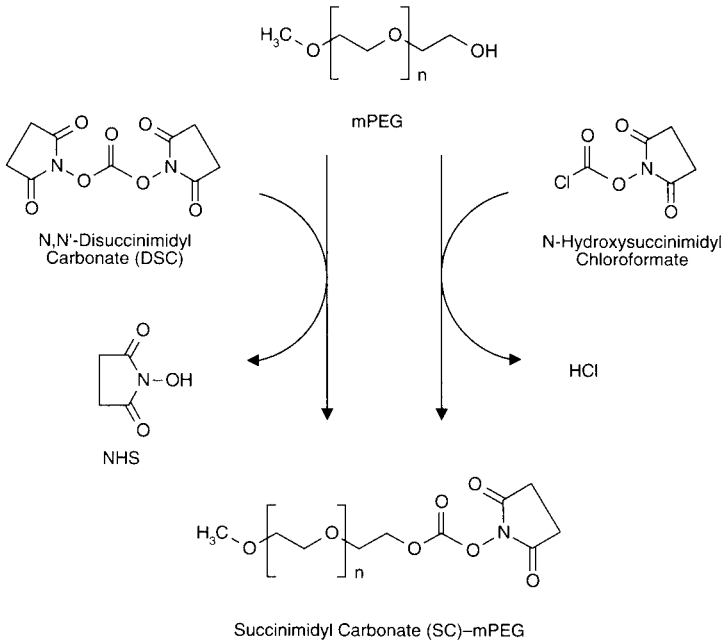


Figure 25.6 An alternative route to an SC derivative of mPEG can be accomplished by the reaction of the terminal hydroxyl group of the polymer with either *N*,*N*′-disuccinimidyl carbonate or *N*-hydroxysuccinimidyl chloroformate.

2. Dissolve 6 mmol of either *N*-succinimidyl chloroformate or *N*,*N*′-disuccinimidyl carbonate (Aldrich) in 10 ml of dry acetone.
3. Dissolve 6 mmol of 4-(dimethylamino)pyridine in 10 ml of dry acetone (base).
4. With stirring, add the solution prepared in step 2 to the PEG solution prepared in step 1. Next, slowly add the solution prepared in step 3.
5. React for 2 hours if activating with *N*-succinimidyl chloroformate or 6 hours if using *N*,*N*′-disuccinimidyl carbonate. Maintain stirring with a magnetic stirring bar.
6. If *N*-succinimidyl chloroformate was used, filter out the white precipitate of 4-(dimethylamino) pyridine hydrochloride using a glass fiber filter pad. Collect the supernatant.
7. For either activation chemistry, precipitate the SC–PEG formed by addition of diethyl ether until no further precipitation is observed (typically 3–4 volumes of solvent).
8. Redissolve the precipitated product in acetone and precipitate again using diethyl ether. Repeat at least once more to remove completely excess reactants.
9. Dry the SC–PEG and store at 4°C.

Protocol for the Coupling of SC–mPEG to Proteins

1. Dissolve the protein to be PEGylated in cold 0.1 M sodium phosphate, pH 7.5, at a concentration of 1–10 mg/ml.

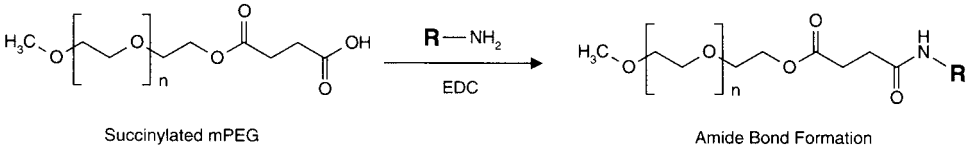


Figure 25.7 Succinylated mPEG derivative may be coupled to amine-containing molecules using a carbodiimide reaction to form an amide bond.

2. With stirring, add a quantity of SC–mPEG to the protein solution at the molar ratio of polymer-to-protein desired. The ratio of activated polymer addition typically is expressed versus the molar quantity of primary amines present on the protein being modified. Ratios of SC–PEG-to-amines between 0.3:1 and 8:1 were investigated by Miron and Wilchek (1993) for the derivatization of egg white lysozyme. The greater the ratio of activated polymer to protein, the higher the molecular weight of the resultant complex. Experiments may have to be done using a number of different reaction ratios to determine the optimal PEGylation level for a particular protein.
3. React overnight at 4°C.
4. Remove excess SC–PEG by dialysis or gel filtration.

1.3. Carbodiimide Coupling of Carboxylate–PEG Derivatives

PEG contains only hydroxyl functional groups in its native state that need to be activated or modified in some manner to allow efficient conjugation to other molecules. These hydroxyls can be modified to possess carboxylates by reaction with anhydride compounds. Acylation of PEG with succinic anhydride or glutaric anhydride gives *bis*-modified products having carboxylates at both ends. Modification of mPEG yields the monosubstituted derivative containing a single carboxylate. Creation of these derivatives was first described by Abuchowski *et al.* (1984). Once the carboxylate–PEG modification is formed, it can be used to couple directly with amine-containing molecules by use of the carbodiimide reaction (Chapter 3, Section 1).

A carbodiimide may be used to activate the carboxylates to highly reactive *o*-acylisourea intermediates. When generated in the presence of an amine-containing protein or other molecule, these active esters will react with the nucleophiles to give amide bond derivatives (Figure 25.7). Atassi and Manshouri (1991) used this technique to PEGylate various peptides. In this instance, the reaction was carried out in dimethylformamide (DMF) due to the solubility of the peptides in this solvent. The organic-soluble carbodiimides dicyclohexyl carbodiimide (DCC) (Chapter 3, Section 1.4) or diisopropyl carbodiimide (DIC) (Chapter 3, Section 1.5) were used to perform the conjugation. However, aqueous phase reactions can be done using this approach just as easily as organic-based conjugations if the water-soluble reagent EDC (1-ethyl-3-(3-dimethylam inopropyl)carbodiimide) is employed (Chapter 3, Section 1.1). The general protocols for using carbodiimides outlined in the referenced sections may be used to conjugate a carboxylate-containing PEG derivative to an amine-containing protein or other molecule. The formation of a carboxylate-containing PEG derivative can be done according to the following protocol (adapted from Atassi and Manshouri, 1991).

Protocol

1. Dissolve 1 g of mPEG (MW 5,000) in 5 ml of anhydrous pyridine by heating to 50°C.
2. To the stirring mPEG solution, add several 0.5 g aliquots of solid succinic anhydride over a period of several hours.
3. React for a further 2 hours at 50°C.
4. Evaporate the pyridine solvent by using a flash evaporator or a rotary evaporator under vacuum.
5. Redissolve the residue in water (the solution may have to be heated to fully dissolve) and again evaporate to dryness. Repeat until the odor of pyridine is nearly gone.
6. Remove remaining reactants by dialysis against water using a membrane having a molecular weight cutoff of 1,000.

1.4. CDI Activation and Coupling

N,*N*′-carbonyldiimidazole (CDI) is a highly reactive carbonylating compound which was first shown to be an excellent amide bond forming agent in peptide synthesis (Paul and Anderson, 1962). Later it was used to activate both carboxylic groups and hydroxyls in the immobilization of amine-containing ligands to prepare affinity chromatography supports (Battling *et al*., 1973; Hearn, 1987).

The activation of a carboxylate group with CDI proceeds to give an intermediate imide with imidazole as the active leaving group. In the presence of a primary amine-containing compound, the nucleophile attacks the electron-deficient carbonyl, displacing the imidazole and forming a stable amide bond.

For hydroxyl-containing compounds, CDI will react to form an intermediate imidazolyl carbamate, which in turn can react with *N*-nucleophiles to give an *N*-alkyl carbamate linkage. Proteins normally couple through their N-terminals (α-amine) and lysine side-chain (ε-amine) functional groups. The final bond is an uncharged, urethane derivative having excellent chemical stability (Figure 25.8). The result of CDI activation of PEG and subsequent coupling to a protein or other amine-containing molecule is a linkage identical to that obtained using SC chemistry, described previously (Section 1.2, this chapter).

CDI-activated PEG is stable for years in a dried state or in organic solvents devoid of water. The activated polymer also will have an excellent half-life to hydrolysis even in the coupling environment. Unlike some activation chemistries that degrade rapidly and have half-lives on the order of minutes, imidazole carbamates have half-lives measured in hours. For instance, an agarose chromatography support activated with CDI will take up to 30 hours at pH 8.5–9.0 for complete loss of activity. The hydrolysis of CDI–PEG derivatives causes the release of CO_2 and imidazole. The hydrolyzed product thus reverts back to the original hydroxylic PEG compound, leaving no residual groups to cause potential sites for nonspecific interactions.

The optimal coupling condition for a CDI–PEG or CDI–mPEG reaction is in an alkaline pH environment, typically above pH 8.5. The coupling reaction proceeds at greatest efficiency when the target molecule is reacted at about 1 pH value above its pI or pK_a. The reaction can be done directly in an organic solvent environment if the molecule to be modified demonstrates poor solubility in aqueous systems. The advantage of an organic coupling reaction is that there is no competing hydrolysis of the active groups, so very high modification yields of PEG can be realized.

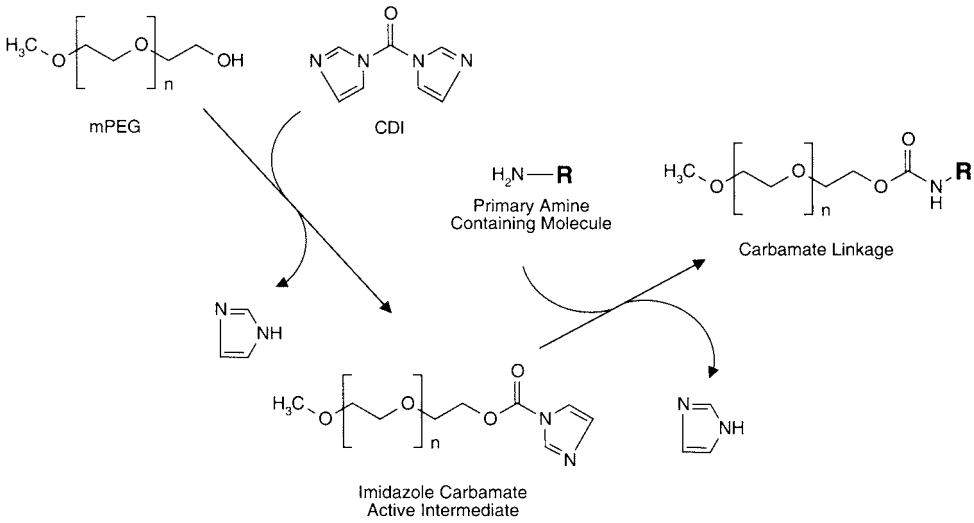


Figure 25.8 *N,N′*-carbonyldiimidazole (CDI) may be used to activate the terminal hydroxyl of mPEG to an imidazole carbamate. Reaction of this intermediate with an amine-containing compound results in the formation of a stable carbamate linkage.

There are a few precautions that should be noted when doing a CDI activation and coupling experiment. First, CDI itself is extremely unstable to aqueous environments, much more so than the active imidazolyl carbamate that's formed after PEG activation. Therefore, the activation step must be done in a solvent that is free of water. If unacceptable amounts of water are present, CDI will be immediately broken down to CO_2 and imidazole. The evolution of bubbles upon addition of CDI to a PEG solution is the telltale sign of high water content. Only freshly obtained solvents analyzed to be extremely low in moisture or those dried over a molecular sieve should be used. A water content of less than 0.1 percent in the solvent is usually all right for a CDI activation procedure.

A second precaution is to carry out the activation step in a fume hood away from sources of ignition. Most CDI activation protocols use flammable or toxic solvents and care should be taken in handling and disposing of them.

The coupling reaction using CDI–mPEG or CDI–PEG derivatives is slower than that obtained using NHS ester or SC-coupling methods. Therefore, the reaction times used with CDI chemistry are typically on the order of 1–2 days at 4°C at a pH of about 8.5. Increasing the pH of the reaction to pH 9 or 10 will speed up the coupling. In addition, doing the reaction at room temperature also helps in this regard. If the molecule to be modified is stable at alkaline pH values and room temperature, then these conditions may be used to decrease the time of the suggested protocol.

The following method is adapted from Beauchamp *et al.* (1983).

Protocol for the Activation of mPEG with CDI

1. Dissolve mPEG (MW 5,000) in dry dioxane at a concentration of 50 mM (0.25 g/ml) by heating to 37°C.

2. Add solid CDI to a final concentration of 0.5 M (81 mg/ml).
3. React for 2 hours at 37°C with stirring.
4. To remove excess CDI and reaction by-products, Beauchamp *et al.* (1983) dialyzed against water at 4°C. However, the imidazole carbamate groups on mPEG formed during the activation process are subject to hydrolysis in aqueous environments. A better method may be to precipitate the activated mPEG with diethyl ether as in the protocol described previously for SC activation (Section 1.2, this chapter).
5. Finally, dry the isolated product by lyophilization (if the water dialysis method is used) or by use of a rotary evaporator (if the ether precipitation method is used).

Protocol for the Coupling of CDI–mPEG to Proteins

1. Dissolve the protein to be PEGylated in 10 mM sodium borate, pH 8.5, at a concentration of 1–10 mg/ml. Higher pH values may be used to increase the reaction rate, for instance, 0.1 M sodium carbonate, pH 9–10.
2. Add CDI–mPEG to this solution with stirring to bring the final concentration of the activated polymer to 180 mM. *Note*: Other ratios of polymer-to-protein may be used, depending on the modification level desired. Some optimization of the derivatization level may have to be done to obtain conjugates having the best amount of polymer substitution with retention of protein activity.
3. React for 48 hours at 4°C. If higher pH or room temperature conditions are used, the reaction time can be decreased to 24 hours.
4. Remove unconjugated mPEG and reaction by-products by dialysis or gel filtration.

1.5. Miscellaneous Coupling Reactions

PEG or mPEG may be conjugated to proteins or other molecules using other coupling chemistries in addition to the ones mentioned in the previous sections. Almost any activation method that can be built off of the terminal hydroxyl(s) of PEG may be employed to PEGylate target molecules. For instance, Bergström *et al.* (1992) created an epoxy derivative of the polymer by reaction with epichlorohydrin under alkaline conditions. The reactive alkyl halogen end of epichlorohydrin first is coupled to the hydroxyls of PEG to give the terminal glycidyl ether derivative (Figure 25.9). The epoxy-functionalized polymer then could be used to covalently modify a poly(ethylene imine)-coated polystyrene surface to prevent nonspecific protein adsorption. This type of derivative also could be used to modify other amine-, hydroxyl-, or sulfhydryl-containing molecules (Chapter 2, Section 4.1).

Creation of a sulfhydryl-reactive PEG derivative was done by Goodson and Katre (1990) by reacting an active ester–maleimide heterobifunctional crosslinker with the amino groups of a PEG-amine polymer (Pillai and Mutter, 1980). An amine-terminal derivative of a discrete PEG-type polymer is available from Thermo Fisher or Quanta BioDesign (Chapter 18). Reaction with the *N*-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzene sulfonic acid results in an amide bond derivative (Figure 25.10). This creates terminal maleimide groups on each PEG-amine molecule. Maleimide compounds can be used in a site-directed coupling

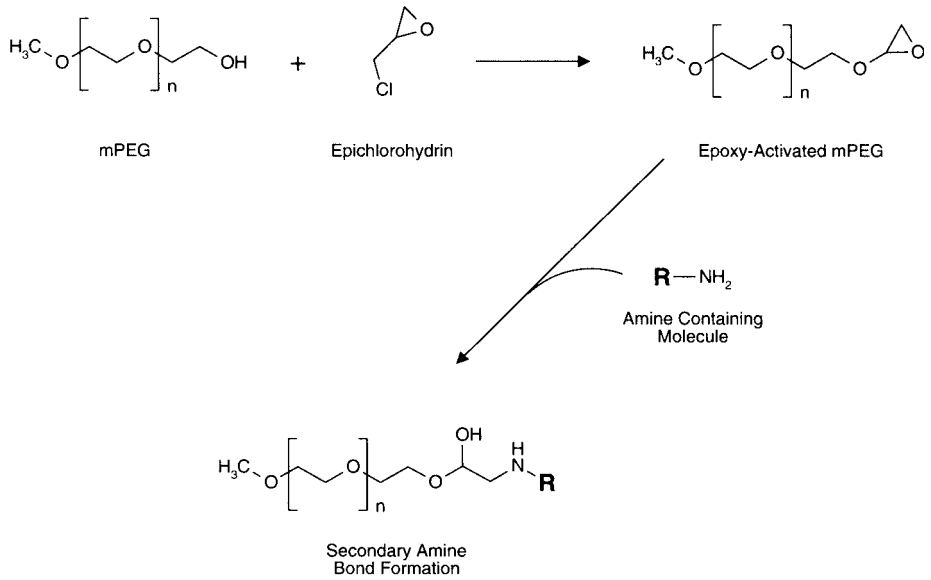


Figure 25.9 Epichlorohydrin can be used to activate the hydroxyl group of mPEG, creating an epoxy derivative. Reaction with amine-containing molecules yields secondary amine bonds.

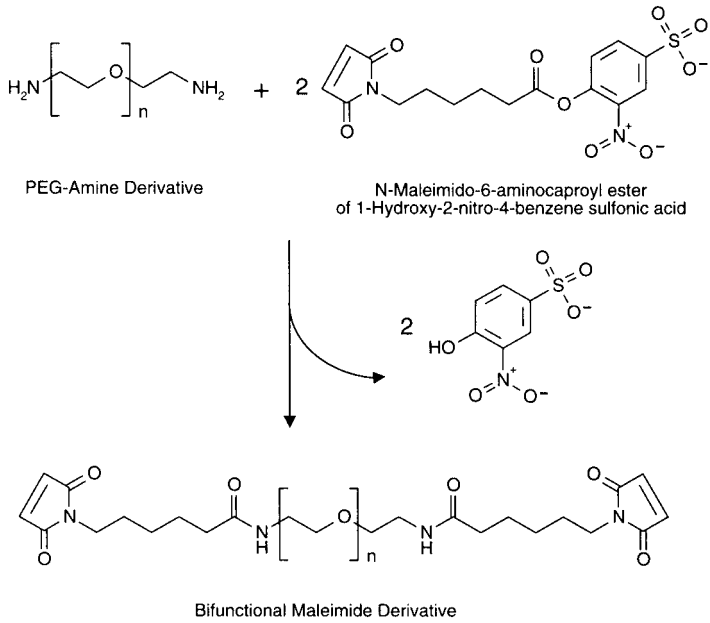
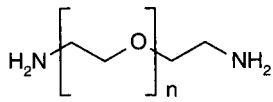


Figure 25.10 PEG-amine compounds may be reacted with this heterobifunctional crosslinker to form amide bond derivatives terminating in maleimide groups. This results in a homobifunctional reagent capable of crosslinking thiol molecules. Subsequent reaction with sulfhydryl-containing molecules yields thioether linkages.

procedure to specifically PEGylate at the sulfhydryl groups of proteins and other molecules (Chapter 2, Section 2.2).



PEG-Amine Derivative
(Jeffamine Series from Texaco;
Various Polymer Lengths Available)

In another approach, Wirth *et al.* (1991) and Chamow *et al.* (1994) transformed the terminal hydroxyl group of mPEG into an aldehyde residue by the Moffatt oxidation procedure (Harris *et al.*, 1984). In this reaction, the hydroxyl is treated with acetic anhydride in dimethyl sulfoxide (DMSO)-containing triethylamine, converting it to the aldehyde (Figure 25.11). After stirring at room temperature for 48 hours, the aldehyde derivative is isolated by precipitation with ether and ethyl acetate. An aldehyde can be conjugated to proteins or other amine-containing molecules by reductive amination using sodium cyanoborohydride (Chapter 3, Section 4). The advantage of an aldehyde–PEG derivative over the other amine-reactive chemistries described previously is that the active function will not hydrolyze or readily degrade before the coupling reaction is initiated. In addition, reductive amination is a reasonably mild conjugation technique well-tolerated by most proteins.

For additional information on PEG-based reagents and coupling chemistry, see Chapter 18, which discusses the unique discrete PEG compounds.

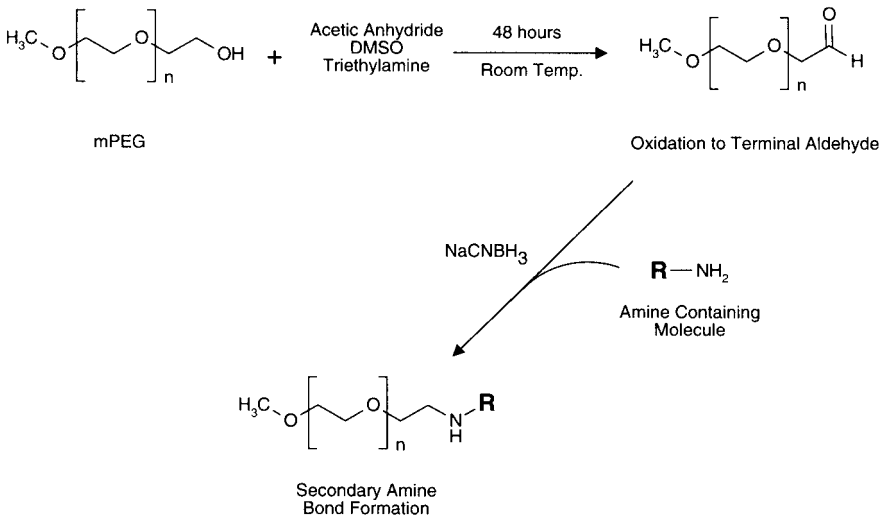
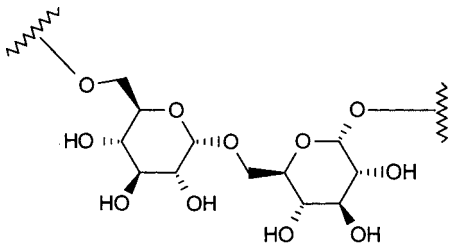


Figure 25.11 A terminal aldehyde function on mPEG may be formed through an oxidative process at elevated temperatures. This derivative may be used to modify amine-containing molecules by reductive amination.

2. Protein Modification with Activated Dextrans

Dextran is a naturally occurring polymer that is synthesized in yeasts and bacteria for energy storage. It is mainly a linear polysaccharide consisting of repeating units of D-glucose linked together in glycosidic bonds (Chapter 1, Section 2), wherein the carbon-1 of one monomer is attached to the hydroxyl group at the carbon-6 of the next residue. This configuration is the same as that found in the α-1,6 linked disaccharide isomaltose. The same disaccharide is found at the branch points of glycogen and amylopectin. Occasional branch points also may be present in a dextran polymer, occurring as α-1,2, α-1,3, or α-1,4 glycosidic linkages. The branch type and degree of branching vary by species.



Isomaltose (a-1,6) Repeating
Unit of Dextran Polymer Chains

The hydroxylic content of the dextran sugar backbone makes the polymer very hydrophilic and easily modified for coupling to other molecules. Unlike PEG, discussed previously, which has modifiable groups only at the ends of each linear polymer, the hydroxyl functional groups of dextran are present on each monomer in the chain. The monomers contain at least 3 hydroxyls (4 on the terminal units) that may undergo derivatization reactions. This multivalent nature of dextran allows molecules to be attached at numerous sites along the polymer chain.

Soluble dextran of molecular weight 10,000−500,000 has been used extensively as a modifying or crosslinking agent for proteins and other molecules. It has been used as a drug carrier to transport greater concentrations of antineoplastic pharmaceuticals to tumor sites *in vivo* (Bernstein *et al.*, 1978; Heindel *et al.*, 1990), as conjugated to biotin to make a sensitive anterograde tracer for neuroatomic studies (Brandt and Apkarian, 1992), as a hapten carrier to illicit an immune response against coupled molecules (Dintzis *et al.*, 1989; Shih *et al.*, 1991), as an inducer of B-cell proliferation by coupling anti-Ig antibodies (Brunswick *et al.*, 1988), as a multifunctional linker to crosslink monoclonal antibody conjugates with chemotherapeutic agents (Heindel *et al.*, 1991), and as a stabilizer of enzymes and other proteins (Zlateva *et al.*, 1988; Nakamura *et al.*, 1990). As is true of PEG conjugates with proteins, dextran modification of macromolecules provides increased circulatory half-life *in vivo*, decreased immunogenicity, and a heat and protease protective effect when coupled at sufficient density (Mumtaz and Bachhawat, 1991).

The following sections describe the major activation and coupling methods used with dextran polymers. The reactive derivatives may be used to couple with proteins and other molecules containing the appropriate functional groups.

2.1. Polyaldehyde Activation and Coupling

The dextran polymer contains adjacent hydroxyl groups on each glucose monomer. These diols may be oxidized with sodium periodate to cleave the associated carbon–carbon bonds and produce aldehydes (Chapter 1, Section 4.4). This procedure results in two aldehyde groups formed per glucose monomer, thus producing a highly reactive, multifunctional polymer able to couple with numerous amine-containing molecules (Bernstein *et al.*, 1978) (Figure 25.12). Polyaldehyde dextran may be conjugated with amine groups by Schiff base formation followed by reductive amination to create stable secondary (or tertiary amine) linkages (Chapter 2, Section 5) (Figure 25.13).

Proteins may be modified with oxidized dextran polymers under mild conditions using sodium cyanoborohydride as the reducing agent. The reaction proceeds primarily through ε-amino groups of lysine located at the surface of the protein molecules. The optimal pH for the reductive amination reaction is an alkaline environment between pH 7 and 10. The rate of reaction is greatest at pH 8–9 (Kobayashi and Ichishima, 1991), reflecting the efficiency of Schiff base formation at this pH.

Polyaldehyde dextran can be used to couple many small molecules, such as drugs, to a targeting molecule like an antibody. The multivalent nature of the oxidized dextran backbone provides more sites for conjugation than possible using direct coupling of the drug with the antibody itself. Similarly, detection molecules such as fluorescent probes can be conjugated in greater amounts using a dextran carrier than is feasible with direct modification of a protein.

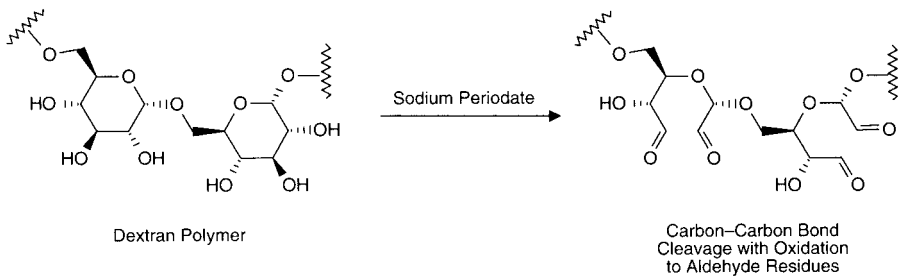


Figure 25.12 Dextran polymers can be oxidized with sodium periodate to create a polyaldehyde derivative. Note that additional oxidation may occur to cleave off another carbon atom and create an aldehyde on the adjacent C—OH group.

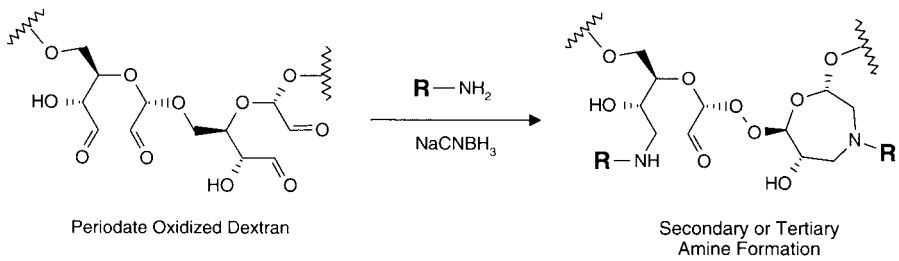


Figure 25.13 Polyaldehyde dextran may be used as a multifunctional crosslinking agent for the coupling of amine-containing molecules. Reductive amination creates secondary amine or tertiary amine linkages.

The following protocol for creating the polyaldehyde dextran derivative is based on the method of Bernstein *et al.* (1978).

Protocol for Oxidizing Dextran with Sodium Periodate

1. Dissolve sodium periodate ($NaIO_4$) (Sigma) in 500 ml of deionized water at a concentration of 0.03 M (6.42 g). Protect from light.
2. Dissolve dextran (Polysciences) of molecular weight between 10,000 and 40,000 in the sodium periodate solution with stirring.
3. React overnight at room temperature in the dark.
4. Remove excess reactant by extensive dialysis against water. The purified polyaldehyde dextran may be lyophilized for long-term storage.

The degree of oxidation may be assessed by measurement of the aldehydes formed. Zhao and Heindel (1991) suggest derivatizing the polyaldehyde dextran with hydroxylamine hydrochloride and measuring the amount of HCl released by titration. However, this may be tedious and time-consuming. A simpler method may be to take advantage of the fact that periodate-oxidized sugars are capable of reducing Cu^{2+} to Cu^{+}, which can be detected using the bicinchoninic acid (BCA) reagent (Thermo Fisher) (Smith, P. S. *et al.*, 1985). The formation of Cu^{+} is in direct proportion to the amount of aldehydes present in the polymer. BCA will form a purple-colored complex with Cu^{+} which can be measured at 562 nm.

Protocol for Coupling Polyaldehyde Dextran to Proteins

1. Dissolve or buffer-exchange the periodate-oxidized dextran in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 10–25 mg/ml. Other buffers having a pH range of 7–10 may be used with success, as long as they do not contain competing amines (such as Tris). A reaction environment of pH 8–9 (0.1 M sodium bicarbonate) will give the greatest yield of reductive amination coupling.
2. Add 10 mg of the protein to be coupled to the dextran solution. Other ratios of dextran-to-protein may be used as appropriate. For instance, if more than one protein or a protein plus a smaller molecule are both to be conjugated to the dextran backbone, the amount of protein added initially may have to be scaled back to allow the second molecule to be coupled latter. Many times, a small molecule such as a drug will be coupled to the dextran polymer first, and then a targeting protein such as an antibody conjugated secondarily. The optimal ratio of components forming the dextran conjugate should be determined experimentally to obtain the best combination possible.
3. In a fume hood, add 0.2 ml of 1 M sodium cyanoborohydride (Aldrich) to each ml of the protein/dextran solution. Mix well. *Caution*: Cyanoborohydride is extremely toxic and should be handled only in well-ventilated fume hoods. Dispose of cyanide-containing solutions according to approved guidelines.
4. React for at least 6 hours at room temperature. Overnight reactions also may be done.
5. To block excess aldehydes, add 0.2 ml of 1 M Tris, pH 8, to each ml of the reaction. *Note*: If a second molecule is to be coupled after the initial protein conjugation, don't block the remaining aldehydes until the second molecule is added.
6. React for an additional 2 hours at room temperature.

7. Purify the protein–dextran conjugate from unconjugated protein and dextran by gel filtration using a column of Sephacryl S-200 or S-300. Small molecules may be removed from a dextran conjugate by dialysis.

2.2. Carboxyl, Amine, and Hydrazide Derivatives

Dextran derivatives containing carboxyl- or amine-terminal spacer arms may be prepared by a number of techniques. These derivatives are useful for coupling amine- or carboxylate-containing molecules through a carbodiimide-mediated reaction to form an amide bond (Chapter 3, Section 1). Amine-terminal spacers also can be used to create secondary reactive groups by modification with a heterobifunctional crosslinking agent (Chapter 5).

This type of modification process has been used to form sulfhydryl-reactive dextran polymers by coupling amine spacers with crosslinkers containing an amine reactive end and a thiol-reactive end (Brunswick *et al.*, 1988; Noguchi *et al.*, 1992). The result was a multivalent sulfhydryl-reactive dextran derivative that could couple numerous sulfhydryl-containing molecules per polymer chain.

Several chemical approaches may be used to form the amine- or carboxyl-terminal dextran derivative. The simplest procedure may be to prepare polyaldehyde dextran according to the procedure of Section 2.1 (this chapter) and then make the spacer arm derivative by reductively aminating an amine-containing organic compound onto it. For instance, short diamine compounds such as ethylene diamine or diaminodipropylamine (3,3′-imino*bis*propylamine) can be reacted in large excess with polyaldehyde dextran to create numerous modifications along the polymer having terminal primary amines. Carboxyl-terminal derivatives may be prepared similarly by coupling molecules such as 6-aminocaproic acid or β-alanine to polyaldehyde dextran. Alternatively, an amine-terminal spacer may be reacted with succinic anhydride to form the carboxylate derivative (Chapter 1, Section 4.2).

Another approach uses reactive alkyl halogen compounds containing a terminal carboxylate group on the other end to form spacer arms off the dextran polymer from each available hydroxyl. In this manner, Brunswick *et al.* (1988) used chloroacetic acid to modify the hydroxyl groups to form the carboxymethyl derivative. The carboxylates then were aminated with ethylene diamine to create an amine-terminal derivative (Inman, 1985). Finally, the amines were modified with iodoacetate to form a sulfhydryl-reactive polymer (Figure 25.14).

In a somewhat similar scheme, Noguchi *et al.* (1992) prepared a carboxylate spacer arm by reacting 6-bromohexanoic acid with a dextran polymer. The carboxylate then was aminated with ethylene diamine to form an amine-terminal spacer (Figure 25.15). This dextran derivative finally was reacted with *N*-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Chapter 5, Section 1.1) to create the desired sulfhydryl-reactive polymer (Section 2.4, this chapter). The SPDP-activated polymer then could be used to prepare an immunoconjugate composed of an antibody against human colon cancer conjugated with the drug mitomycin-C.

Hydrazide derivatives also may be prepared from a periodate-oxidized dextran polymer or from a carboxyl-containing dextran derivative by reaction with *bis*-hydrazide compounds (Chapter 4, Section 8). A hydrazide terminal spacer provides reactivity toward aldehyde- or ketone-containing molecules. Thus, the hydrazide–dextran polymer can be used to conjugate specifically glycoproteins or other polysaccharide-containing molecules after they have been oxidized with periodate to form aldehydes (Chapter 1, Section 4.4).

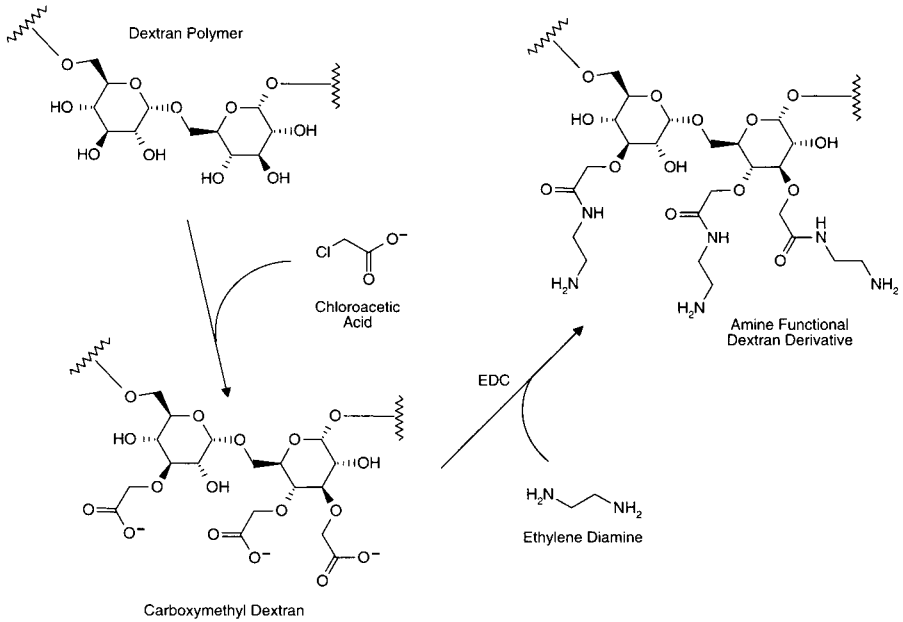


Figure 25.14 An amine derivative of dextran may be prepared through a two-step process involving the reaction of chloroacetic acid with the hydroxyl groups of the polymer to create carboxylates. Next, ethylene diamine is coupled in excess using a carbodiimide-mediated reaction to give the primary amine functional groups.

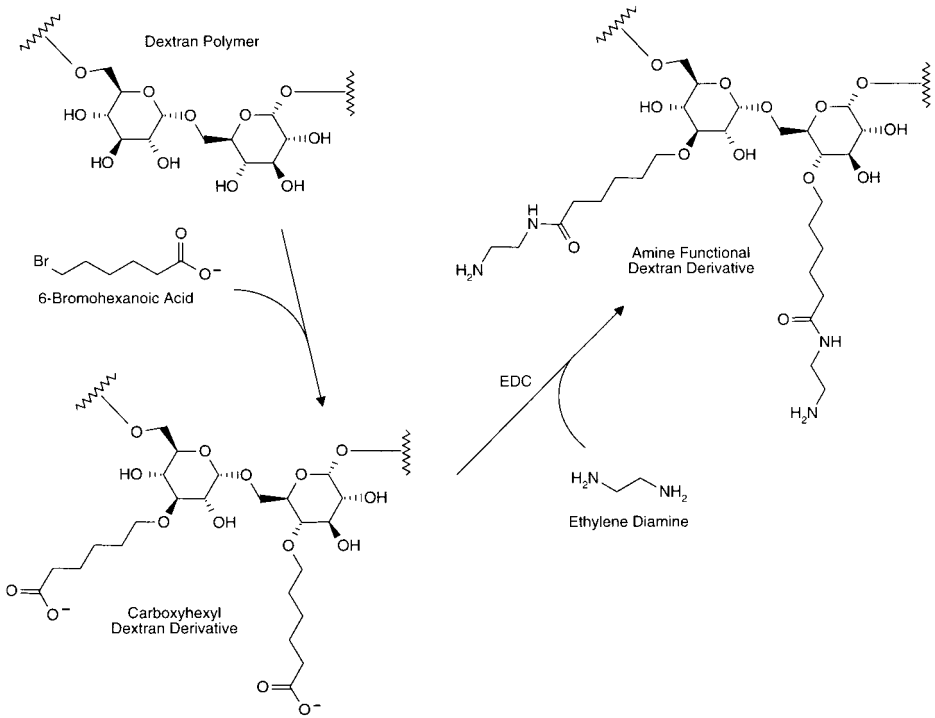


Figure 25.15 Amino-dextran derivatives may be prepared by the reaction of 6-bromohexanoic acid with the hydroxyl groups of the polymer followed by coupling of ethylene diamine using EDC.

The following protocols may be used to create carboxyl-, amine-, or hydrazide-containing derivatives of dextran.

Preparation of Amine or Hydrazide Derivatives by Reductive Amination

1. Prepare polyaldehyde dextran according to the method of Section 2.1 (this chapter).
2. To make an amine derivative of dextran, dissolve ethylene diamine (or another suitable diamine) in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, at a concentration of 3 M. *Note*: Use of the hydrochloride form of ethylene diamine is more convenient, since it avoids having to adjust the pH of the highly alkaline free-base form of the molecule. Alternatively, to prepare a hydrazide–dextran derivative, dissolve adipic acid dihydrazide (Chapter 4, Section 8.1) in the coupling buffer at a concentration of 30 mg/ml (heating under a hot water tap may be necessary to completely dissolve the hydrazide compound). Adjust the pH to 7.2 with HCl and cool to room temperature.
3. Dissolve polyaldehyde dextran in the ethylene diamine (or adipic dihydrazide) solution at a concentration of 25 mg/ml.
4. In a fume hood, add 0.2 ml of 1 M sodium cyanoborohydride to each ml of the diamine/dextran solution. Mix well. *Caution*: Cyanoborohydride is extremely toxic and should be handled only in well-ventilated fume hoods. Dispose of cyanide-containing solutions according to approved guidelines.
5. React for at least 6 hours at room temperature. Overnight reactions also may be done.
6. Remove excess diamine and reaction by-products by dialysis.

The ethylene diamine–dextran derivative may be used for the coupling of carboxylate-containing molecules by the carbodiimide reaction, for the coupling of amine-reactive probes, or to modify further using heterobifunctional crosslinkers. The hydrazide–dextran derivative may be used to crosslink aldehyde-containing molecules, such as oxidized carbohydrates or glycoproteins.

Protocol for the Modification of Dextran with Chloroacetic Acid

1. In a fume hood, prepare a solution consisting of 1 M chloroacetic acid in 3 N NaOH.
2. Immediately add dextran polymer to a final concentration of 40 mg/ml. Mix well to dissolve.
3. React for 70 minutes at room temperature with stirring.
4. Stop the reaction by adding 4 mg/ml of solid NaH_2PO_4 and adjusting the pH to neutral with 6 N HCl.
5. Remove excess reactants by dialysis.

The carboxymethyl–dextran derivative may be used to couple amine-containing molecules by the carbodiimide reaction. Heindel *et al.* (1994) prepared the lactone derivative of carboxymethyl–dextran by refluxing for 5 hours in toluene or other anhydrous solvents. The lactone derivative is highly reactive toward amine-containing molecules, thus creating a preactivated polymer for conjugation purposes.

2.3. Epoxy Activation and Coupling

Epoxy activation of hydroxylic polymers is commonly used as a means to immobilize molecules on solid phase chromatographic supports that contain hydroxyl groups (Sundberg and

Porath, 1974). *Bis*-oxirane compounds also can be used to introduce epoxide groups into soluble dextran polymers in much the same manner (Böldicke *et al.*, 1988; Böcher *et al.*, 1992). The epoxide group reacts with nucleophiles in a ring-opening process to form a stable covalent linkage. The reaction can take place with primary amines, sulfhydryls, or hydroxyl groups to create secondary amine, thioether, or ether bonds, respectively (Chapter 2, Section 1.7).

Modification of dextran polymers with 1,4-butanediol diglycidyl ether results in ether derivatives of the dextran hydroxyl groups, which then contain hydrophilic spacers with terminal epoxy functions (Figure 25.16).

Protocol

1. In a fume hood, mix 1 part 1,4-butanediol diglycidyl ether with 1 part 0.6 N NaOH containing 2 mg/ml sodium borohydride.
2. With stirring, add 5 mg of dextranto each ml of the *bis*-epoxide solution. Mix well to dissolve.

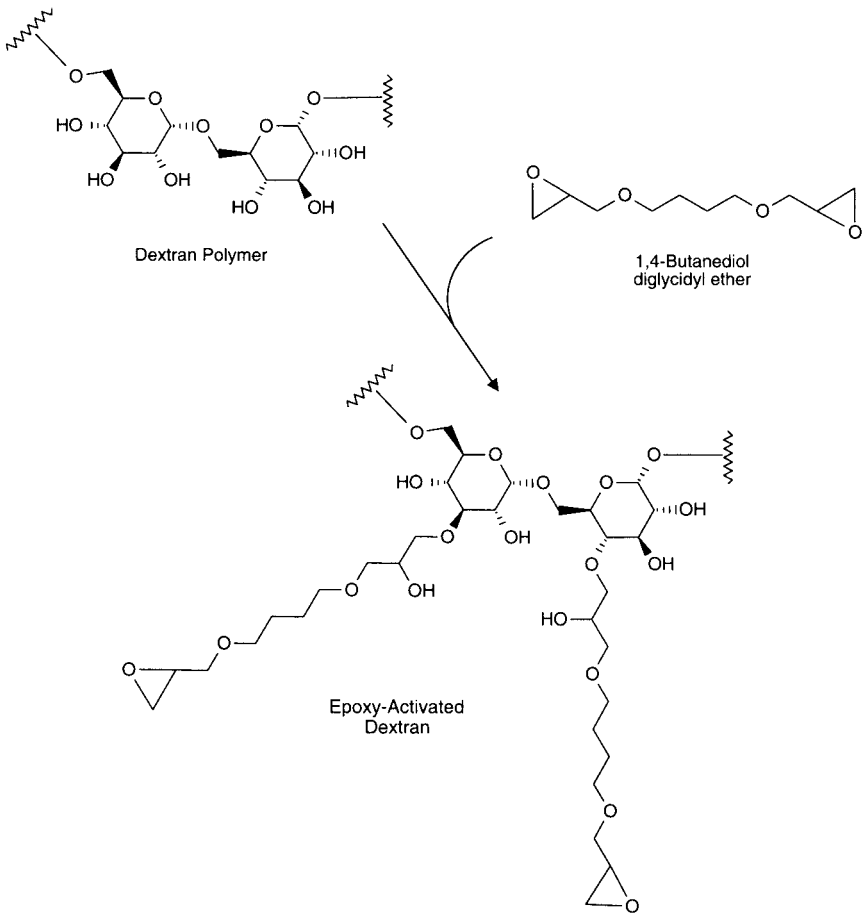


Figure 25.16 An epoxy-functional dextran derivative may be prepared by the reaction of 1,4-butanediol diglycidyl ether with the hydroxyl groups of the polymer.

3. React for 12 hours at 25°C or 3–4 hours at 37°C.
4. Extensively dialyze the solution against water to remove excess reactants. The activated dextran may be lyophilized for long-term storage.

The epoxide-activated dextran may be used to conjugate amine-, sulfhydryl-, or hydroxyl-containing molecules. The reaction of the epoxide groups with hydroxyls requires high pH conditions, usually in the range of pH 11–12. Amine nucleophiles react at more moderate alkaline pH values, typically needing buffer environments of at least pH 9–10. Sulfhydryl groups are the most highly reactive nucleophiles with epoxides, requiring a buffered system closer to the physiological range, pH 7.5–8.5, for efficient coupling.

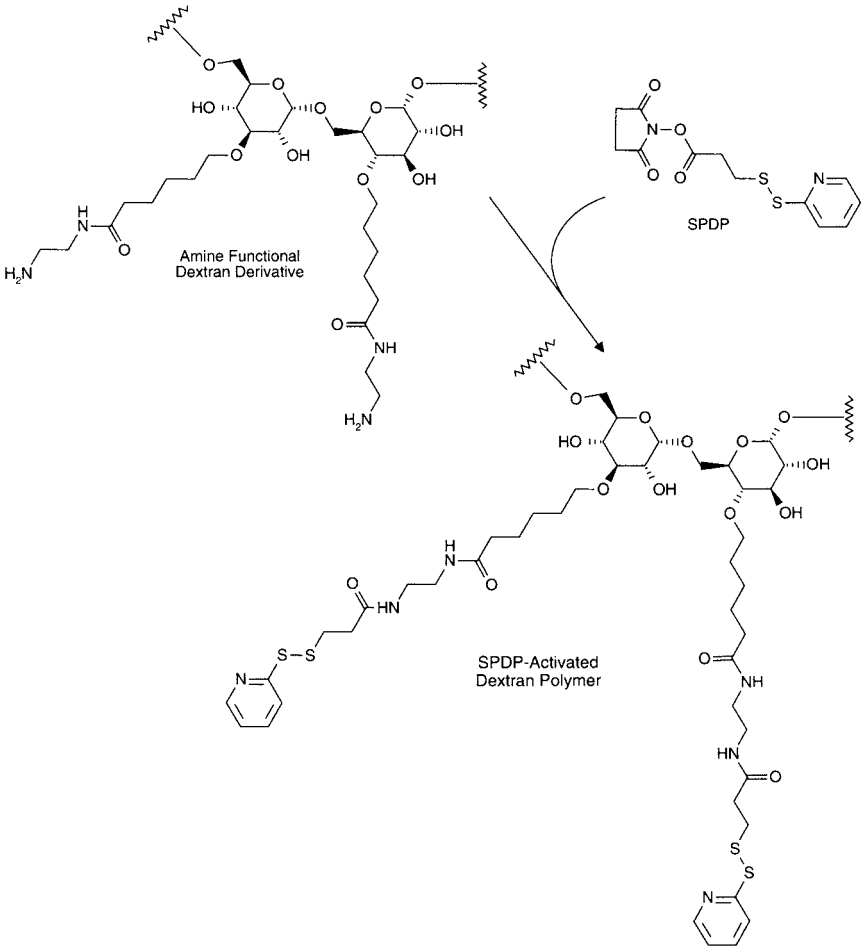


Figure 25.17 An amine-functionalized dextran derivative may be further reacted with SPDP to create a sulfhydryl-reactive product.

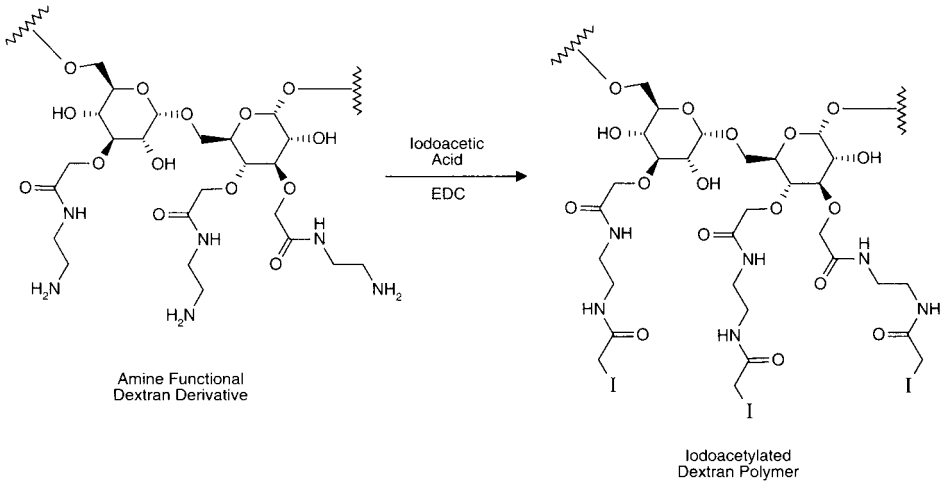


Figure 25.18 An amine-derivative of dextran may be coupled with iodoacetic acid using a carbodiimide reaction to produce a sulfhydryl-reactive iodoacetamide polymer.

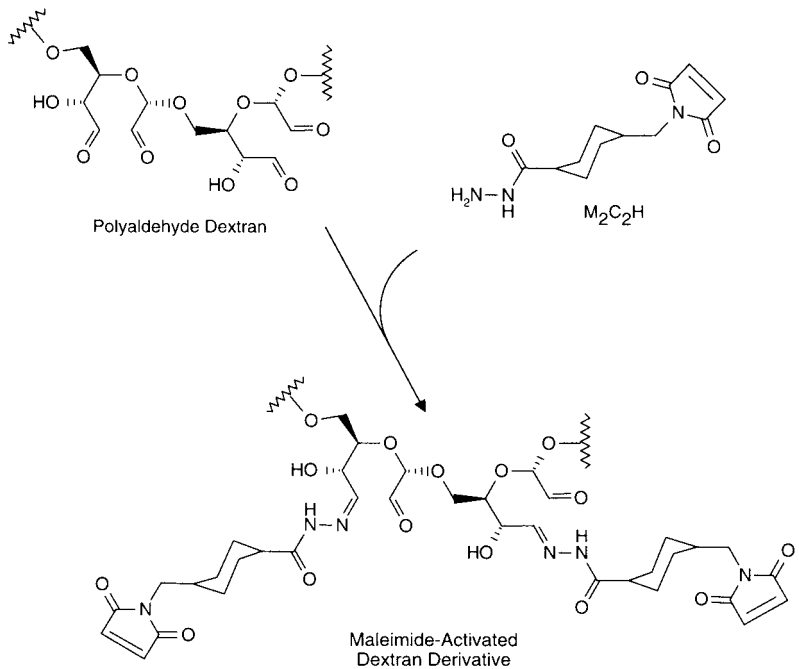


Figure 25.19 Polyaldehyde dextran may be modified with the hydrazide end of M2C2H to create a thiol-reactive polymer.

2.4. Sulfhydryl-Reactive Derivatives

Sulfhydryl-reactive dextran derivatives may be prepared through the use of heterobifunctional crosslinking agents (Chapter 5). In particular, crosslinkers containing pyridyl disulfide, maleimide, or iodoacetyl groups on one end are quite effective in directing a conjugation reaction to thiols. Both maleimide and iodoacetyl activation procedures will yield nonreversible bonds with sulfhydryl-containing molecules. Pyridyl disulfide compounds, by contrast, react with thiols to form cleavable disulfide bonds that can be reversed by reduction.

Noguchi *et al.* (1992) used an amine-terminal spacer arm derivative of dextran to react with SPDP (Chapter 5, Section 1.1) in the creation of a pyridyl disulfide-activated polymer (Figure 25.17). Brunswick *et al.* (1988) used a different amine-terminal spacer arm derivative of dextran and subsequently coupled iodoacetate to form a sulfhydryl-reactive polymer (Figure 25.18). Heindel *et al.* (1991) used a unique approach. They modified polyaldehyde dextran with a heterobifunctional crosslinker containing a hydrazide group on one end and a maleimide group on the other (Chapter 5, Section 2). The hydrazides reacted with the aldehyde groups to form hydrazone linkages, leaving the maleimide ends free to result in a thiol-reactive dextran derivative (Figure 25.19).

26

Enzyme Modification and Conjugation

Enzymes are widely used in bioconjugate chemistry as detection components in assay systems. The catalytic activity of an enzyme can be used to turn substrate molecules into chromogenic, fluorescent, or chemiluminescent products, which are easily detectable or quantifiable by imaging, microscopy, or spectroscopy. If an enzyme is conjugated to a targeting molecule specific for some analyte of interest, then an assay system can be constructed to localize or measure the analyte. The most common targeting molecule is an antibody having antigen-binding specificity for the substance to be measured. An enzyme conjugated to such an antibody can be used to visualize the presence of antigen. Due to the advantages of this simple concept, enzyme linked immunoadsorbent assays (ELISAs) have become the most important type of immunoassay system available.

The rapid turnover rate of some enzymes allows ELISAs to be designed that surpass the sensitivity of radiolabeling techniques. In addition, substrates can be chosen to produce soluble products that can be accurately quantified by their absorbance or fluorescence. Alternatively, substrates are available which form insoluble, highly colored precipitates, excellent for localizing antigens in blots, cells, or tissue sections. The flexibility of enzyme-based assay systems makes the chemistry of enzyme conjugation one of the most important application areas in bioconjugate techniques.

The following sections briefly describe the principal enzymes used for conjugation with other protein molecules, particularly in the design of ELISA and other immunoassay systems.

1. Properties of Common Enzymes

1.1. Horseradish Peroxidase

HRP (donor:hydrogen peroxide oxidoreductase; EC 1.11.1.7), derived from horseradish roots, is a enzyme of molecular weight 40,000 that can catalyze the reaction of hydrogen peroxide with certain organic, electron-donating substrates to yield highly colored products. The reaction of HRP with its fundamental substrate, H_2O_2, forms a stable intermediate that can dissociate in the presence of a suitable electron donor, oxidizing the donor and potentially creating a color change. The donor can consist of oxidizable molecules like ascorbate, cytochrome C,

ferrocyanide, or the leuco forms of many dyes. A large variety of electron-donating dye substrates is commercially available for use as HRP detection reagents. Some of them can be used to form soluble colored products for use in spectrophotometric detection systems, while other substrates form insoluble products that are especially apropos for staining techniques. In addition, substrates are available that create fluorescent or chemiluminescent products upon oxidation with HRP. The chemiluminescent substrates are among the most sensitive of all detection reagents, facilitating the detection of as little as attogram quantities of many targeted analytes. The pH optimum for HRP is 7.0.

HRP is a hemoprotein containing photohemin IX as its prosthetic group. The presence of the heme structure gives the enzyme its characteristic color and maximal absorptivity at 403 nm.The ratio of its absorbance in solution at 403 nm to its absorbance at 275 nm, called the RZ or Reinheitzahl ratio, can be used to approximate the purity of the enzyme. However, at least seven isoenzymes exist for HRP (Shannon *et al*., 1966; Kay *et al*., 1967; Strickland *et al*., 1968), and their RZ values vary from 2.50 to 4.19. Thus, unless the RZ ratio is precisely known or determined for the particular isoenzyme of HRP utilized in the preparation of an antibody–enzyme conjugate, subsequent measurement after crosslinking would yield questionable results in the determination of the amount of HRP present in the conjugate.

HRP is a glycoprotein that contains significant amounts of carbohydrate. Its polysaccharide chains are often used in crosslinking reactions. Mild oxidation of its associated sugar residues with sodium periodate generates reactive aldehyde groups that can be used for conjugation to amine-containing molecules. Reductive amination of oxidized HRP to antibody molecules in the presence of sodium cyanoborohydride is perhaps the simplest method of preparing highly active conjugates with this enzyme (Chapter 3, Section 1.4 and Chapter 20, Section 1.3).

Other methods of HRP conjugation include the use of the homobifunctional reagent glutaraldehyde (Chapter 4, Section 6.2) and the heterobifunctional crosslinker, SMCC (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (Chapter 5, Section 1.3). Using glutaraldehyde, a two-step protocol usually is employed to limit the extent of oligomer formation. Nevertheless, this method often causes unacceptable amounts of precipitated conjugate. Despite this disadvantage, glutaraldehyde conjugation is still routinely used, especially in the preparation of some antibody–enzyme reagents that go into established diagnostic assays. The use of the N-hydroxysuccinimide (NHS) ester–maleimide crosslinker, SMCC, provides better control over the conjugation process. SMCC usually is reacted first with HRP to create a derivative containing sulfhydryl-reactive maleimide groups. The maleimide-activated enzyme can be purified and freeze-dried, providing a ready source of modified HRP to react with a sulfhydryl-containing antibody. Several preactivated forms of this enzyme are available from Thermo Fisher.

The size of HRP is an advantage in preparing antibody–enzyme conjugates, since the overall complex size also can be designed to be small. Relatively low-molecular-weight conjugates are able to penetrate cellular structures better than large, polymeric complexes. This is why HRP conjugates are often the best choice for immunocytochemical staining techniques. Small conjugate size means greater accessibility to antigenic structures within tissue sections.

Another distinctive advantage of HRP is its robust nature and stability, especially under the conditions employed for crosslinking. HRP is stable for years in a freeze-dried state, and the purified enzyme can be stored in solution at 4°C for many months without significant loss of activity. The enzyme also retains excellent activity after being modified with a conjugation reagent or periodate-oxidized to form aldehyde groups on its polysaccharide chains. Depending

on the methods used for crosslinking, HRP conjugates can be constructed to have a high ratio of enzyme-to-antibody or a low ratio—both retaining high specific activity.

The disadvantages associated with HRP are several. The enzyme only contains two available primary ε-amine groups—extraordinarily low for most proteins—thus limiting its ability to be activated with amine-reactive heterobifunctionals. HRP is sensitive to the presence of many antibacterial agents, especially azide. It also is reversibly inhibited by cyanide and sulfide (Theorell, 1951). Finally, while the enzymatic activity of HRP is extremely high, its useful lifespan or practical substrate development time is somewhat limited. After about an hour of substrate turnover, in some situations its activity can be decreased severely.

Nevertheless, HRP is by far the most popular enzyme used in antibody–enzyme conjugates. One survey of enzyme use stated that HRP is incorporated in about 80 percent of all antibody conjugates, most of them utilized in diagnostic assay systems.

1.2. Alkaline Phosphatase

Alkaline phosphatases [AP, orthophosphoric-monoester phosphorylase (alkaline optimum); EC 3.1.3.1] represent a large family of almost ubiquitous isoenzymes found in organisms from bacteria to animals. In mammals, there are two forms of AP, one form present in a variety of tissues and another form found only in the intestines. They share common attributes in that the phosphatase activity is optimal at pH 8–10, is activated by the presence of divalent cations, and is inhibited by cysteine, cyanides, arsenate, various metal chelators, and phosphate ions. Most conjugates created with AP utilize the form isolated from calf intestine.

AP isoenzymes can cleave associated phosphomonoester groups from a wide variety of substrates. The exact biological function of these enzymes is not well understood. They can behave *in vivo* in their classic phosphohydrolase role at alkaline pH, but at neutral pH AP isoenzymes can act as phosphotransferases. In this sense, suitable phosphate acceptor molecules can be utilized in solution to increase the reaction rates of AP on selected substrates. Typical phosphate acceptor additives include diethanolamine, Tris, and 2-amino-2-methyl-1propanol. The presence of these additives in substrate buffers can dramatically increase the sensitivity of AP ELISA determinations, even when the substrate reaction is done in alkaline conditions.

Calf intestinal AP has a molecular weight of about 140,000. The active site of AP contains two zinc atoms and a single magnesium atom, both of which are essential for activity (Kim and Wyckoff, 1991). Substrate development with AP thus should be done in buffered environments containing small concentrations of these divalent cations to maintain optimal active site conformation. Avoid the presence of metal chelators such as EDTA, since they may extract these ions from the enzyme and inhibit activity. The pH optimum for APs can vary from pH 8–0, depending on the type of isoenzyme. Calf intestinal AP peaks in activity at the higher pH values of this range, and substrate reactions are commonly performed in diethanolamine buffer at pH 9.8. The calf intestinal enzyme has the highest catalytic rate constant yet discovered for AP isoenzymes, 3,500 s^{-1}.

Purified preparations of calf intestinal AP maintained in solution are usually stored in the presence of a stabilizer, which is typically 3 M NaCl. The enzyme also may be lyophilized, but it may experience activity loss with each freeze-thaw cycle. AP is not stable under acidic conditions. Lowering the pH of an AP solution to 4.5 reversibly inhibits the enzyme. It is recommended that all handling, storage, and use of AP be done under conditions >pH 7.0 to maintain the highest possible catalytic activity.

AP often is a difficult enzyme to work with when preparing enzyme conjugates. Activity losses may occur upon modification with a crosslinking agent or after coupling to an antibody molecule. Simply following established protocols for making antibody–AP conjugates doesn't always assure retention of enzyme activity. Sometimes activity losses can be traced to particular batches or to certain suppliers of the enzyme. Using a highly purified, high activity AP preparation helps to maintain good resultant activity in the conjugate.

Ironically, AP is the enzyme of choice for some applications due to its stability. Since it can withstand the moderately high temperatures associated with hybridization assays better than HRP, AP often is the enzyme of choice for labeling oligonucleotide probes. AP also is capable of maintaining enzymatic activity for extended periods of substrate development. Increased sensitivity can be realized in ELISA procedures by extending the substrate incubation time to hours and sometimes even days. These properties make AP the second most popular choice for antibody–enzyme conjugates (behind HRP), being used in almost 20 percent of all commercial enzyme-linked assays.

Conjugation methods typically employed with AP include glutaraldehyde-mediated crosslinking (Chapter 4, Section 6.2) and the use of the heterobifunctional reagents, SMCC (Chapter 5, Section 1.3) or SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate) (Chapter 5, Section 1.1). Heterobifunctional crosslinkers provide the best control over the crosslinking process and typically result in antibody–enzyme conjugates of high activity. Many conjugation protocols incorporate a sodium phosphate buffer system to block reversibly the AP active site during chemical modification. This prevents derivatization from occurring in the catalytic site, thus better retaining activity in the resultant conjugate.

1.3. β-Galactosidase

β-Galactosidase (β-Gal; β-D-galactoside galactohydrolase; EC 3.2.1.23; also called lactase) catalyzes the hydrolysis of β-D-galactoside in the presence of water to galactose and alcohol. This type of enzyme is found widespread in many microorganisms, plants, and animals. β-Gal can be used to determine lactose in biological fluids and it is employed in food processing operations, particularly in immobilized form. β-Gal also has good characteristics when conjugated to antibody molecules for use in ELISA systems (Wallenfels and Weil, 1972; Byrne and Johnson, 1975; Kato *et al*., 1975a, b).

β-Gal has a molecular weight of 540,000 and is composed of four identical subunits of MW 135,000, each with an independent active site (Melchers and Messer, 1973). The enzyme has divalent metals as cofactors, with chelated Mg^{+2} ions required to maintain active site conformation. The presence of NaCl or dilute solutions (5 percent) of low-molecular-weight alcohols (methanol, ethanol, etc.) causes enhanced substrate turnover. β-Gal contains numerous sulfhydryl groups and is glycosylated.

Commercially available β-gal usually is isolated from *Escherichia coli* and has a pH optimum at 7-7.5. By contrast, mammalian β-galactosidases usually have a pH optimum within the range of 5.5–6; thus, interference from endogenous β-gal during immunohistochemical staining can be avoided.

Due to the relatively high-molecular-weight of the enzyme, conjugates formed with antibodies and β-gal tend to be much bulkier than those associated with AP or horseradish peroxidase. For this reason, antibody conjugates made with β-gal may have more difficulty penetrating tissue structures during immunohistochemical staining techniques than those made with the other enzymes.

Although numerous research articles have been written describing the preparation and use of antibody conjugates with β-gal, the enzyme remains a minor player in ELISA procedures. Less than 1 percent of all commercial ELISA products utilize this enzyme.

β-Gal may be conjugated to antibody molecules using the heterobifunctional reagent SMCC. This crosslinker is reacted first with an antibody through its amine-reactive NHS ester end to form a maleimide-activated derivative. This is in contrast with most antibody–enzyme conjugation schemes utilizing SMCC, wherein the enzyme is modified first and a sulfhydryl-containing antibody is coupled secondarily. However, since β-gal already contains abundant free sulfhydryl residues that can participate in coupling to a maleimide-activated protein, conjugations with this enzyme often are done with the antibody being the first component modified. This route avoids having to create sulfhydryls on the antibody molecule, either by reduction or modification with a thiolating reagent. Thus, antibody–β-gal conjugates usually are simpler to make than using other enzymes.

1.4. Glucose Oxidase

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4; GO) is a flavoenzyme that catalyzes the oxidation of β-D-glucose to δ-D-gluconolactone. The intermediate product of the catalysis is a reduced enzyme–$FADH_2$ complex that in the presence of oxygen gets oxidized back to enzyme–FAD with release of hydrogen peroxide. The enzyme consists of two identical subunits (MW 80,000 each) bound together by disulfide linkages (O'Malley and Weaver, 1972). GO contains two tightly bound flavin adenine dinucleotide (FAD) cofactors, one per subunit, which are critical to its oxidoreductase activity. Each subunit also contains one molecule of chelated iron. The intact protein consists of about 74 percent amino acids, 16 percent neutral carbohydrate, and 2 percent amino-sugars (total molecular weight 160,000). GO operates under a relatively broad pH range of 4–7, but its pH optimum is 5.5. The commercially available preparation of GO is typically isolated from *Aspergillus niger.*

Glucose oxidase is widely used in diagnostic assays for the determination of glucose concentration in physiological fluids. Detectability of the oxidation products is done through an enzyme-coupled reaction wherein liberated H_2O_2 is reacted with peroxidase and a suitable chromogenic substrate. The development of substrate color thus is proportional to the amount of H_2O_2 released which is in turn related to the amount of glucose originally present. The production of hydrogen peroxide also can be quantified using a luminescence procedure with luminol to produce light in proportion to the glucose concentration (Williams *et al.*, 1976).

GO often is used in solution phase reactions as well as being immobilized on "dip-sticks" and electrodes. Although its overall clinical usage is widespread, its use as conjugated to antibodies in enzyme-linked assay systems is minor compared to the popularity of other enzymes like horseradish peroxidase and AP. Of the total number of commercial diagnostic assays utilizing antibody–enzyme conjugates, GO is employed in less than 1 percent of clinical tests. The enzyme remains, however, an important tool in many assays developed for research use. One particular advantage to the enzyme is that there is no endogenous GO activity in mammalian tissues, making it an excellent choice for immunohistochemical staining procedures.

Antibody conjugates with GO can be made using the crosslinking agents glutaraldehyde (Chapter 4, Section 6.2) or SMCC (Chapter 5, Section 1.3). The heterobifunctional reagent SMCC provides the best control over the conjugation process and usually results in high-activity preparations.

2. Preparation of Activated Enzymes for Conjugation

Enzymes may be modified to contain reactive groups useful for conjugation with other proteins. This operation may be done using homobifunctional (Chapter 4) or heterobifunctional (Chapters 5, 17, and 18) reagents that can covalently couple to some chemical target on the enzyme and result in a terminal reactive group that can crosslink with another molecule. Enzyme activation also may take advantage of the presence of polysaccharide constituents—oxidizing them with sodium periodate to form reactive aldehydes.

Whatever the method of conjugate creation, the most important considerations are retention of activity in the complex and prevention of extensive oligomer generation, which may cause precipitation. The following methods discuss some of the more common methods for producing enzyme conjugates. The list, however, is by no means inclusive of every possible procedure used in the literature.

2.1. Glutaraldehyde-Activated Enzymes

Glutaraldehyde is a homobifunctional crosslinker containing an aldehyde residue at both ends of a 5-carbon chain. Its primary reactivity is toward amine groups, but the reaction may occur by more than one mechanism. As discussed in Chapter 4, Section 6.2, glutaraldehyde is able to form Schiff base linkages with amines that can be reduced with sodium cyanoborohydride to create stable secondary amine linkages. However, glutaraldehyde also exists as α, β-unsaturated polymers in aqueous solution. The double bonds of these polymers can undergo an addition reaction with amines that results in covalent bond formation even without a reductant being present. Fresh glutaraldehyde may contain little polymer formation. However, the older the preparation of glutaraldehyde, the more likely it is that it contains appreciable amounts of polymer. Thus, reactions with this crosslinking agent can result in indistinct conjugation products.

The high reactivity of glutaraldehyde also makes it difficult to control the conjugation process. Proteins crosslinked with this reagent often form substantial amounts of precipitated products due to polymerization. The degree of oligomer formation can be moderated somewhat by using a two-step protocol, but the first protein activated with the molecule still can form large molecular weight complexes.

Despite the obvious disadvantages of glutaraldehyde-mediated conjugation, the crosslinker continues to be used to form enzyme–antibody complexes and in other applications. Many diagnostic tests still utilize antibody–enzyme conjugates prepared through glutaraldehyde crosslinking procedures.

The one- and two-step procedures for enzyme activation and conjugation using glutaraldehyde can be found in Chapter 20, Section 1.2.

2.2. Periodate Oxidation Techniques

Molecules containing polysaccharide chains may be oxidized to possess reactive aldehyde residues by treatment with sodium periodate. Any adjacent carbon atoms containing hydroxyl groups will be affected, cleaving the carbon–carbon bond and transforming the hydroxyls into

aldehydes. Glycoproteins may be oxidized in this manner to form reactive intermediates useful for crosslinking procedures involving reductive amination (Chapter 3, Section 4). This conjugation technique can direct the coupling process away from polypeptide active regions, thus helping to preserve catalytic activity or avoid binding sites.

Enzymes that contain carbohydrate, such as HRP or GO, may be oxidized with periodate to create reactive derivatives that subsequently can be used to label antibodies or other targeting molecules at their amine groups. The aldehyde-HRP intermediate may be stored for extended periods in a frozen or lyophilized state without loss of activity (either enzymatic or coupling potential). Avoid, however, storage in a liquid state, since polymerization may occur—resulting in precipitation and loss of activity.

The protocols for periodate oxidation of HRP and its conjugation with other proteins may be found in Chapter 20, Section 1.3.

2.3. SMCC-Activated Enzymes

The heterobifunctional crosslinker SMCC, or its water-soluble analog sulfo-SMCC, can be used to activate enzymes through their amines, leaving terminal maleimide groups on the protein surface (Chapter 5, Section 1.3). The NHS ester end of the crosslinker reacts with ε-lysine or N-terminal amines to form amide bonds. The maleimide end of the reagent is stable enough in aqueous solution to allow purification of the activated enzyme prior to conjugation with a second protein. The maleimide group can react with sulfhydryl groups to create thioether linkages. A maleimide-activated enzyme may be stored in a lyophilized state for extended periods without loss of sulfhydryl-coupling capability.

The use of this type of heterobifunctional reagent allows controlled conjugations to take place, precisely regulating the exact ratio of each protein in the final complex and the size of the resultant conjugate. In addition, the second-stage conjugation through sulfhydryl groups provides directed coupling at discrete sites within a protein molecule, thus providing the potential to better avoid active centers or binding regions. For instance, antibody molecules can be coupled to enzymes in their hinge region after mild disulfide reduction to effect the crosslink in an area away from the antigen binding site.

Protocols for the activation of enzyme molecules with SMCC (or sulfo-SMCC) can be found in Chapter 20, Section 1.1. Conjugates formed using this method usually result in high-activity complexes giving excellent sensitivity for use in immunoassays or other applications.

2.4. Hydrazide-Activated Enzymes

Hydrazide groups can react with carbonyl groups to form stable hydrazone linkages. Derivatives of proteins formed from the reaction of their carboxylate side chains with adipic acid dihydrazide (Chapter 4, Section 8.1) and the water-soluble carbodiimide EDC (Chapter 3, Section 1.1) create activated proteins that can covalently bind to formyl residues. Hydrazide-modified enzymes prepared in this manner can bind specifically to aldehyde groups formed by mild periodate oxidation of carbohydrates (Chapter 1, Section 4.4). These reagents can be used in assay systems to detect or measure glycoproteins in cells, tissue sections, or blots (Gershoni *et al.*, 1985).

Other molecules can be used in this type of assay approach. Hydrazide-modified (strept)avidin, lectins, biocytin, fluorescent probes and other detectable molecules can be used to detect specifically glycoconjugates in biological samples (Wilchek and Bayer, 1987).

The activation of enzymes using adipic acid dihydrazide and EDC is identical to the procedure outlined for the modification of (strept)avidin (Chapter 23, Section 5). Alternatively, hydrazide groups may be created on enzymes using the heterobifunctional chemoselective reagents described in Chapter 17, Section 2.

2.5. SPDP-Activated Enzymes

SPDP is a heterobifunctional crosslinker containing an NHS ester on one end and a pyridyl disulfide group on the other end (Chapter 5, Section 1.1). The NHS ester end can be used to modify amine groups on enzymes, forming amide bonds. The result of this procedure is to create sulfhydryl-reactive pyridyl disulfide groups on the surface of each enzyme molecule that are able to complex with thiol-containing proteins and other molecules. SPDP-activated enzymes may be purified and stored for extended periods without breakdown of the coupling capacity. The reaction with a sulfhydryl group forms a reversible disulfide linkage that can be cleaved with reducing agents.

The two-step nature of SPDP crosslinking provides control over the conjugation process. Complexes of defined composition can be constructed by adjusting the ratio of enzyme to secondary molecule in the reaction as well as the amount of SPDP used in the initial activation. The use of SPDP in conjugation applications is extensively cited in the literature, perhaps making it one of the more popular crosslinkers available. It is commonly used to form immunotoxins, antibody–enzyme conjugates, and enzyme-labeled DNA probes. A standard activation and coupling procedure can be found in Chapter 5, Section 1.1.

3. Preparation of Biotinylated Enzymes

Biotinylated enzymes can be used as detection reagents in (strept)avidin–biotin assay procedures. Particularly, in the bridged avidin–biotin (BRAB) approach or the ABC technique (Chapter 23, Section 2), a biotin-labeled enzyme is used as the signaling agent after the binding to an antigen of a biotinylated antibody and an (strept)avidin bridging molecule. The biotins on the surface of the enzyme can bind with extraordinary affinity to (strept)avidin–antibody complexes, providing near-covalent interaction potential with high specificity.

Adding a biotin label to an enzyme molecule is simple, given the wide variety of options available. A biotinylation reagent is chosen that has a reactive group that will couple to functional groups on the enzyme (Chapter 11; Chapter 18, Section 3). For instance, NHS-LC-biotin can be used to modify amine groups—a good choice for most biotinylation procedures involving proteins (Chapter 11, Section 1). When free sulfhydryls are present, as in β-gal, a thiol-reactive biotin label may be more appropriate, such as biotin–BMCC (Chapter 11, Section 2). However, a better choice may be to use hydrophilic biotinylation reagents containing a PEG spacer arm, which results in better solubility of the biotinylated enzyme (Chapter 18, Section 3). The protocols for labeling proteins with these reagents can be found in Chapters 11 and 18.

27

Nucleic Acid and Oligonucleotide Modification and Conjugation

Molecular biology techniques incorporating highly sensitive detection methods involving fluorescence, chemiluminescence, or chromogenic enzyme substrates are used widely for assaying oligonucleotide interactions. A major factor in the development of assay and detection systems for RNA and DNA measurement is the ability to modify a nucleic acid with a detectable component while not affecting base pairing. The attachment of a small label, such as a fluorescent molecule, or a large catalytic enzyme to an oligonucleotide probe forms the basis for constructing sensitive hybridization reagents that can be used to detect genomic sequences. Unfortunately, the methods developed to crosslink or label proteins do not always apply to the modification of nucleic acids. The major reactive sites on proteins involve primary amines, sulfhydryls, carboxylates, or phenolates—groups that are familiar and relatively easy to derivatize. RNA and DNA contain none of these functionalities. They also are unreactive with many of the common electrophilic bioconjugate reagents discussed in Part II.

To modify the unique chemical groups on nucleic acids, novel methods have been developed that allow derivatization through discrete sites on the available bases, sugars, or phosphate groups (see Chapter 1, Section 3 for a discussion of RNA and DNA structure). These chemical methods can be used to add a functional group or a label to an individual nucleotide or to one or more sites in oligonucleotide probes or full-sized DNA or RNA polymers.

If an individual nucleotide is modified in the appropriate way, various enzymatic techniques can be used to polymerize the derivative into an existing oligonucleotide molecule. Alternatively, nucleotide polymers can be treated with chemical activators that can facilitate the attachment of a label at particular reactive sites. Thus, there are two main approaches to modifying DNA or RNA molecules: enzymatic or chemical. Both procedures can produce highly active conjugates for sensitive assays to quantify or localize the binding of an oligo probe to its complementary strand in a complex mixture.

The following sections describe the major enzymatic and chemical modification procedures used to label nucleic acids and oligonucleotides.

1. Enzymatic Labeling of DNA

Enzymatic techniques can employ a variety of DNA or RNA polymerases to add controlled amounts of modified nucleotides to an existing stand. However, the most common procedures utilize either DNA polymerase I or terminal deoxynucleotide transferase. The polymerase is used with a template to add modified nucleoside triphosphates to the end of a DNA molecule or to various sites within the middle of a sequence. The terminal transferase can add modified monomers to the 3′ end of a chain without a template.

Three main procedures of enzyme labeling make use of a DNA polymerase: (a) random-primed labeling, (b) nick translation, and (c) polymerase chain reaction (PCR). In random-primed labeling, modified nucleoside triphosphates are added to a DNA template using a random mixture of hexa-deoxynucleotides to serve as 3′-OH primers. The form of polymerase used is the Klenow fragment which lacks the 5′-3′ exonuclease activity of intact *E. coli* DNA polymerase I (Feinberg and Vogelstein, 1983, 1984; Kessler *et al.*, 1990). This method is a simple way of tagging probes prepared from a restriction digest template with randomly incorporated, labeled nucleotides.

Nick-translation labeling involves the use of a dual enzyme system acting on double-stranded DNA (Rigby *et al.*, 1977; Langer *et al.*, 1981; Höltke *et al.*, 1990). The enzymes pancreatic deoxyribonuclease I (DNase I) and *E. coli* DNA polymerase I act in tandem on a DNA helix to incorporate labeled nucleotides into the sequence. DNase I is capable of breaking phosphodiester bonds in intact DNA double-stranded molecules. If it is used in the presence of magnesium ions, it limits the hydrolysis caused by the enzyme to a single strand at a time within the DNA helix. If DNase I is further restricted in the amount added to a reaction, the number of breaks caused in the double helix can be controlled. The addition of DNA polymerase I and the appropriate labeled and unlabeled nucleotide monomers causes the breaks to be filled as quickly as they form. Since a quantity of labeled nucleoside triphosphates is present during the reaction, the labels get incorporated and the parent DNA strands are modified.

Enzymatic labeling of DNA by use of PCR techniques perhaps provides the most powerful way of not only adding a label, but also of amplifying the labeled polymer to produce numerous copies of itself. First invented by Mullis (who went on to win the Nobel Prize; see Saiki *et al.*, 1985, 1988), PCR utilizes heat-stable forms of DNA polymerase, for example the commonly employed *Taq* polymerase isolated from thermophilic eubacterium (*Thermus aquaticus*). The stability of the enzyme allows repeated elevated-temperature denaturations of target DNA, followed by hybridization of two primers onto the single strands. *Taq* DNA polymerase then creates a complementary sequence to the two single strands by elongation of the primers. If repeated cycles of denaturation, hybridization, and elongation are done, the result is an exponential amplification of the original DNA strands (for a review of PCR, see Innis *et al.*, 1990). Labeling of these amplified strands can be accomplished by one of two routes: using labeled primers or using labeled deoxynucleoside triphosphates. Either way, the *Taq* polymerase incorporates the labels into the growing DNA copies of each PCR cycle.

Enzymatic labeling using any of these polymerase methods results in derivatized nucleoside triphosphates being incorporated at numerous locations within an oligonucleotide strand. These modifications potentially can interfere with the hybridization of a probe to a complementary sequence, especially if the level of labeling is high. Enzymatic labeling using terminal transferase is a way to avoid derivatization in the middle of a strand, and thus preserve

sequence or targeting specificity. The enzyme is able to add deoxyribonucleotides to the 3′-OH ends of existing DNA probes without the need for a template. Since modification is limited to a single end of the oligonucleotide, the probe sequence is not disturbed by labeling groups that could possibly prevent hydrogen bonding interactions between base pairs.

Terminal transferase labeling was originally developed using radiolabeled (typically ^{32}P) nucleoside triphosphates (Roychoudhury *et al.*, 1979; Tu and Cohen, 1980). Later, the technique was extended to the use of nonradioactive nucleotide derivatives (Kumar *et al.*, 1988).

Regardless of the type of enzymatic labeling used, it is important that the label be incorporated into the nucleoside triphosphates or primers in a way that does not affect enzyme recognition and activity. Thus, every enzymatic labeling procedure for modifying RNA or DNA probes must start with chemical derivatization of individual nucleotides. Of the many chemical procedures that can be used to modify a nucleoside triphosphate monomer, there are only a few that will result in a derivative still able to be enzymatically added to an existing oligonucleotide strand.

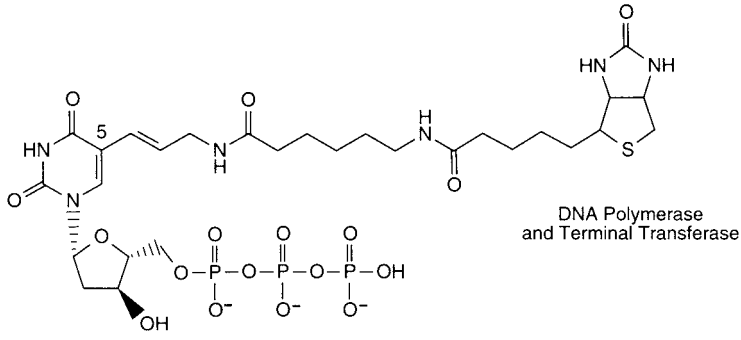
Of the purine nucleosides, dATP may be derivatized at its N-6 position using a long linker arm terminating in a detectable group without losing the ability to be enzymatically incorporated into DNA probes. By contrast, if modification is done at the C-8 position of purine bases, DNA polymerase cannot by used to add the labeled monomer to an existing strand. C-8 derivatives, however, can be added at the 3′ terminal using terminal transferase enzyme.

The pyrimidine nucleosides dUTP or dCTP can be modified at their C-5 position with a spacer arm containing a tag, such as a biotin group, and still remain good substrates for DNA polymerase. Enzymatic labeling with a biotin-modified pyrimidine nucleoside triphosphate is one of the most common methods of adding a detectable group to an existing DNA strand.

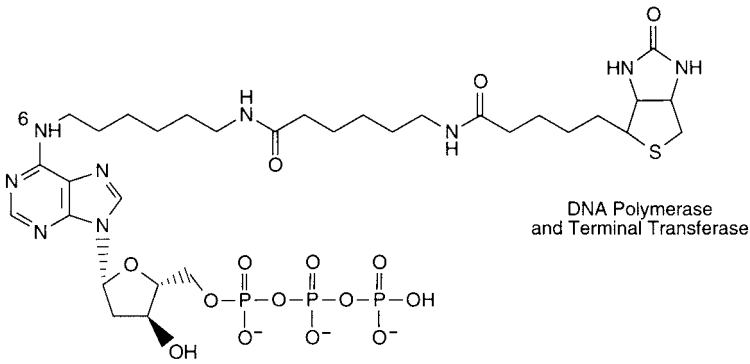
Figure 27.1 illustrates some of the common nucleoside triphosphate derivatives that can be used in enzymatic labeling processes. The following sections describe procedures for enzymatic labeling using nick translation, random prime labeling, and 3′ tailing with terminal transferase. For a review of these methods in greater detail, see Kricka (1992), or Keller and Manak (1989). See Section 2 (this chapter) for a discussion of the chemical methods that can be used to label individual nucleic acids for incorporation into oligonucleotides by enzymatic means.

Protocol for the Labeling of DNA by Nick Translation

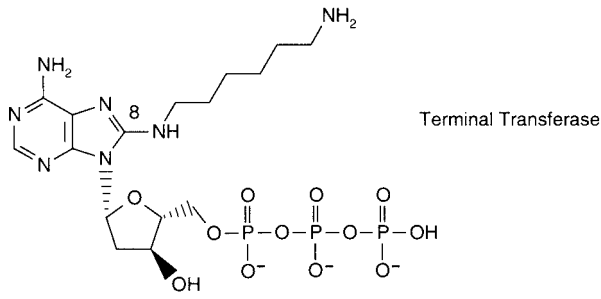
1. In a tube kept cold on ice, add 10 µl of 10 × nick-translation buffer (0.5 M Tris, 0.1 M $MgCl_2$, 0.08 M 2-mercaptoethanol, pH 7.5, containing 0.5 mg/ml bovine serum albumin, BSA), 0.5 µg of double-stranded probe DNA to be labeled, 1 µl of DNase I at a concentration of 2 ng/ml, 1 µl each of 3 types of unmodified deoxynucleoside triphosphates (dNTPs at 100 µM concentration), 1 µl of a labeled dNTP (at 300 µM), 32 µl water. Then add 1 µl of DNA polymerase containing 5–10 units of activity.
2. React for 1 hour at 15°C.
3. Quench the reaction by the addition of 4 µl of 0.25 M EDTA, 2 µl of 10 mg/ml tRNA, and 150 µl of 10 mM Tris, pH 7.5.
4. Purify the labeled DNA from excess reactants by precipitation. Add 20 µl of 4 M LiCl and 500 µl of ethanol (chilled to −20°C). Mix well.
5. Store at −20°C for 30 minutes, and then separate the precipitated DNA by centrifugation at 12,000 g.



Biotin–11–dUTP
Derivatized at the C-5 Position



Biotin–14–dATP
Derivatized at the N-6 Position



8–Aminohexyl–dATP
Derivatized at the C-8 Position

Figure 27.1 Three common nucleoside triphosphate derivatives that can be incorporated into oligonucleotides by enzymatic means. The first two are biotin derivatives of pyrimidine and purine bases, respectively, that can be added to an existing DNA strand using either polymerase or terminal transferase enzymes. Modification of DNA with these nucleosides results in a probe detectable with labeled avidin or streptavidin conjugates. The third nucleoside triphosphate derivative contains an amine group that can be added to DNA using terminal transferase. The modified oligonucleotide then can be labeled with amine-reactive bioconjugation reagents to create a detectable probe.

6. Remove the supernatant and wash the pellet with 70 and 100 percent ethanol, centrifuging after each wash.
7. Re-dissolve the labeled DNA pellet in water and store at −20°C until used.

Protocol for the Labeling of DNA by Random Priming

1. Denature 1 μg of probe DNA (single stranded) with 5 μg of random hexanucleotide primers by boiling for 5 minutes and then rapidly chill on ice. Incubate at least 10 minutes to allow the primers to hybridize to random sites within the probe DNA.
2. Add to a tube on ice, 5 μl of 10× random priming buffer (0.5 M Tris, 0.1 M $MgCl_2$, 10 mM 2-mercaptoethanol, pH 6.6, containing 0.5 mg/ml BSA), 1 μl each of 3 types of unmodified deoxynucleoside triphosphates (dNTPs at 100 μM concentration), 1 μl of a labeled dNTP (at 100 μM), plus 48 μl of water. Then add 2 μl of DNA polymerase (5–10 units).
3. Combine the probe DNA/hexanucleotide preparation with the reaction solution and incubate for 2 hours at 37°C.
4. Quench the reaction by the addition of 2 μl of 0.5 M EDTA, 2 μl of 10 mg/ml tRNA, and 150 μl of 10 mM Tris, pH 7.5.
5. Purify the labeled probe by ethanol precipitation according to steps 4–7 of the protocol previously described for nick translation.

Protocol for Labeling DNA at the 3′ End Using Terminal Transferase

1. Prepare 1 μg of purified DNA probe, either by restriction digestion or by synthetic means.
2. Add to the purified probe, (a) 20 μl of 0.5 M potassium cacodylate, 5 mM $CoCl_2$, 1 mM dithiothreitol (DTT), pH 7.0, (b) 100 μM of a modified deoxynucleoside triphosphate, 4 μl of 5 mM dCTP, and 100 μl of water. Mix.
3. Add terminal transferase to a final concentration of 50 units in the reaction mixture.
4. React for 45 minutes at 37°C.
5. Isolate the labeled probe by alcohol precipitation as described previously for nick translation.

2. Chemical Modification of Nucleic Acids and Oligonucleotides

The chemical modification of nucleic acids at specific sites within individual nucleotides or within oligonucleotides allows various labels to be incorporated into DNA or RNA probes. This labeling process can produce conjugates having sensitive detection properties for the localization or quantification of oligo binding to a complementary strand using hybridization assays.

Some form of chemical labeling process must be used regardless of whether the final oligo conjugate is created by enzymatic or strictly chemical means. If enzymatic modification is to be done, the initial label still must be incorporated into an individual nucleoside triphosphate, which then is polymerized into an existing oligonucleotide strand (Section 1, this chapter). Fortunately, many useful modified nucleoside triphosphates are now available from commercial sources, often eliminating the need for custom derivatization of individual nucleotides.

Chemical modification also may be used to label directly an oligonucleotide, eliminating the enzymatic step altogether. The chemical modification of nucleic acids can encompass several

strategies. The initial derivatization only might be done to add a spacer arm to a particular reactive group on the nucleotide structure. The spacer typically contains a terminal functional group, such as an amine, that can be used to couple another molecule. A secondary modification might be to add a fluorescent tag to the end of the spacer, thus creating a detectable complex. The spacer also may be used to react with a crosslinking agent, such as a heterobifunctional compound (Chapter 5), that can facilitate the conjugation of a protein or another molecule to the modified nucleotide. It should be noted that if enzymatic methods are used to incorporate a small spacer into an oligonucleotide, subsequent chemical conjugation steps still will be needed to add a small label or protein tag.

In some cases, if an oligonucleotide contains the appropriate functional group, a label may be directly incorporated into it using chemical methods. For instance, certain fluorescent molecules or biotin tags can be used to modify nucleotides without going through an initial derivatization step with a spacer arm. Such labels usually contain nucleophilic or photoreactive groups that can couple directly to the oligo using an intermediate activating agent or by photolyzing with UV light, respectively.

Many of the chemical derivatization methods employed in these strategies involve the use of an activation step that produces a reactive intermediary. The activated species then can be used to couple a molecule containing a nucleophile, such as a primary amine or a thiol group. The following sections describe the chemical modification methods suitable for derivatizing individual nucleic acids as well as oligonucleotide polymers.

2.1. Diamine or Bis-Hydrazide Modification of DNA

One of the more useful chemical modifications that can be done on nucleic acids or oligonucleotides is to add an amine-terminal spacer arm using a diamine compound. The resultant amine derivative can be targeted by numerous amine-reactive crosslinkers or modification reagents to create a detectable conjugate. A similar approach can be used to modify a DNA probe with a bis-hydrazide compound (Chapter 4, Section 8) to produce terminal hydrazide group. The oligonucleotide derivative then can be coupled with aldehyde-containing molecules to form conjugates. The following methods utilize activation reagents which transform a particular site on nucleic acids into an amine-reactive or hydrazide-reactive intermediate. Coupling a diamine or bis-hydrazide compound to these activated species results in the formation of an alkyl spacer arm terminating in a primary amine group or a hydrazide functional group, respectively.

Conjugation via Bisulfite Activation of Cytosine

Single-stranded DNA molecules can react with sodium bisulfite, adding a sulfonate group across the 5,6-double bond of cytidine bases and creating 6-sulfo-cytosine derivatives. The reaction catalyzes the deamination of cytosine to uracil by loss of the 4-amino group. Subsequent loss of HSO_3^- effectively forms uracil bases (Figure 27.2). This reaction sequence was recognized in the early 1970s as potential evidence for the mutagenicity of bisulfite (Shapiro *et al.*, 1973, 1974). Shapiro and Weisgras (1970) demonstrated that the bisulfite reaction also can cause transamination to occur at the N-4 position of cytosine. In the presence of an amine-containing molecule, such as a diamine, sodium bisulfite will cause the exchange of the N-4 amine for another amine-containing compound, effectively forming a new covalent linkage

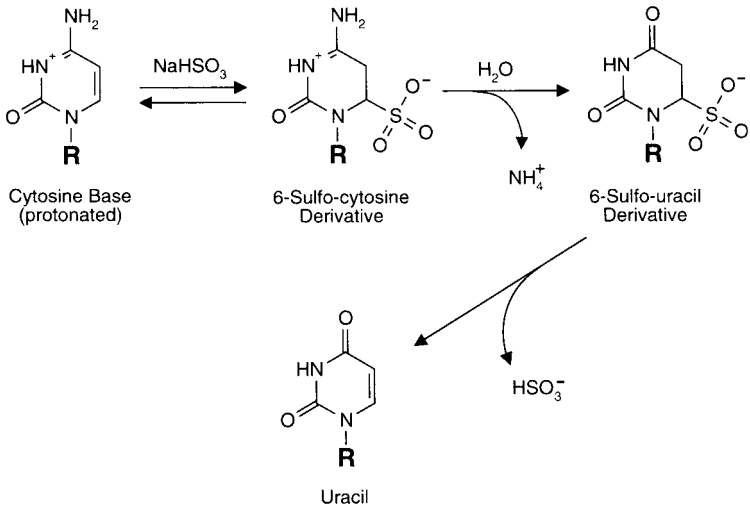


Figure 27.2 Treatment of cytosine bases with bisulfite results in a multi-step deamination reaction, ultimately leading to uracil formation.

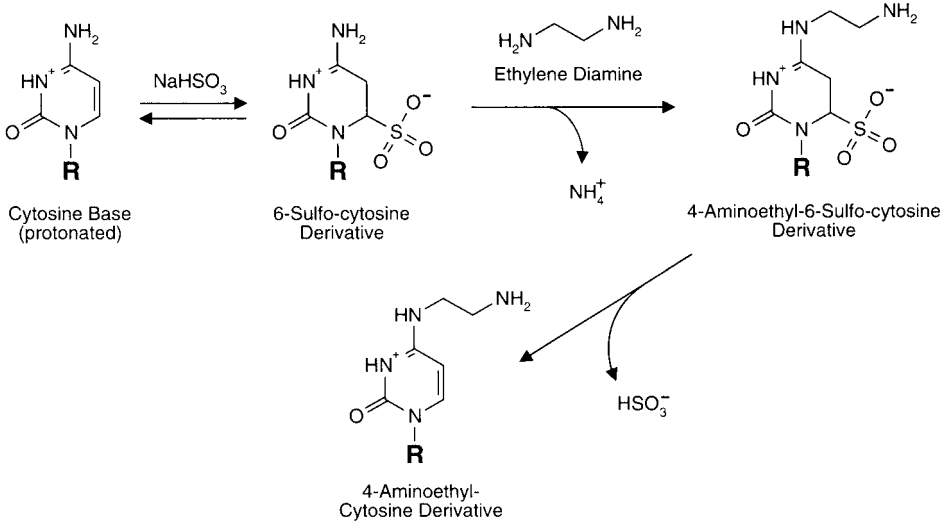


Figure 27.3 The reaction of cytosine with bisulfite in the presence of an excess of an amine nucleophile (such as a diamine compound) leads to transamination at the N-4 position. This process is a route to adding an amine functional group to cytosine residues in oligonucleotides.

with release of ammonium ion (Figure 27.3). Draper and Gold (1980) used this reaction to produce primary amine groups on a poly(C) oligonucleotide by coupling 1,3-diaminopropane to a limited number of the cytosine residues. The amine derivative subsequently could be used to couple a fluorescent probe to the polymer, allowing sensitive studies of messenger RNA.

Bisulfite-catalyzed transamination also can be used to label oligonucleotide probes for application in nonradioisotopic hybridization assays. Viscidi *et al.* (1986) described a method for derivatizing cytosine groups in DNA probes using the short spacer, ethylenediamine. Other diamine molecules also may be used, such as 1,3-diaminopropane, 1,6-diaminohexane, or 3,3′-iminobispropylamine. The use of the long, hydrophilic Jeffamine molecules (Texaco Chemical Co; see Chapter 1, Section 4.3) may be especially well suited for this type of modification due to the presence of a hydrophilic polyethylene glycol (PEG)-based spacer. Longer spacer arms may provide better steric accommodation for larger detection components without interfering substantially in the probe's ability to hybridize to a complementary DNA strand.

If an amine-containing fluorescent probe or hydrazide-containing compound is transaminated onto an oligonucleotide using bisulfite, the labeling of nucleic acids can be done in a single step. An example of this approach is the coupling of biotin hydrazide (Chapter 11, Section 3) to cytosine residues, resulting in a biotinylated oligonucleotide suitable for (strept)avidin-based detection systems (Reisfeld *et al.*, 1987) (Chapter 23).

Since the site of modification on cytosine bases is at a hydrogen bonding position in double helix formation, the degree of bisulfite derivatization should be carefully controlled. Reaction conditions such as pH, diamine concentration, and incubation time and temperature affect the yield and type of products formed during the transamination process. At low concentrations of diamine, deamination and uracil formation dramatically exceed transamination. At high concentrations of diamine (3 M), transamination can approach 100 percent yield (Draper and Gold, 1980). Ideally, only about 30–40 bases should be modified per 1,000 bases to assure hybridization ability after derivatization.

Bisulfite modification of cytosine residues also can be used to add permanently a sulfone group to the C-6 position. In this scheme, the sulfone functions as a hapten recognizable by specific anti-sulfone antibodies. At high concentrations of bisulfite and in the presence of methylhydroxylamine, cytosines are transformed into N^4-methoxy-5,6-dihydrocytosine-6-sulphonate derivatives (Herzberg, 1984; Nur *et al.*, 1989). Labeled antibodies can then be used to detect the hybridization of such probes.

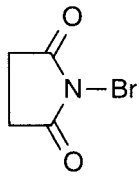
Protocol for Labeling Nucleic Acids by Bisulfite-Catalyzed Transamination

1. Prepare single-stranded DNA (denatured) at a concentration of 1 mg/ml.
2. Prepare bisulfite modification solution consisting of: 3 M concentration of a diamine (i.e., ethylenediamine), 1 M sodium bisulfite, pH 6. The use of the dihydrochloride form of the diamine avoids having to adjust the pH down from the severe alkaline pH of the free-base form. *Note*: The optimum pH for transaminating biotin–hydrazide to cytosine residues using bisulfite is 4.5 (see Section 2.3, this chapter).
3. Add 20 µl of the DNA to 180 µl of bisulfite modification solution. Mix well.
4. React for 3 hours at 42°C.
5. Dialyze the solution against water overnight at 4°C to remove excess reactants.
6. The modified DNA may be recovered by alcohol precipitation according to the method in Section 1 (this chapter) described previously for nick-translation modification. Alternatively, dialysis or gel filtration may be done to remove excess reactants.

Conjugation via Bromine Activation of Thymine, Guanine, and Cytosine

The nucleotide bases of DNA and RNA can be activated with bromine to produce reactive intermediates capable of coupling to nucleophiles (Traincard *et al.*, 1983; Sakamoto *et al.*,

1987; Keller *et al.*, 1988). Bromination occurs at the C-8 position of guanine residues and the C-5 of cytosine, yielding reactive derivatives which can be used to couple diamine spacer molecules by nucleophilic substitution (Figure 27.4). Other pyrimidine derivatives also are reactive to bromine compounds, but adenine residues are more resistant. However, even AMP can be immobilized through the introduction of an aminohexyl spacer at the C-8 position using bromination (Lowe, 1979). Either an aqueous solution of bromine or the compound *N*-bromosuccinimide can be used for this reaction. The alkaline modification proceeds rapidly, but may be too severe for RNA molecules. Coupling of amine-containing molecules is done at elevated temperatures (50°C) to assure good incorporation. Both amine-bearing spacers and probes may be coupled using this strategy. Moreover, the sites of derivatization using bromine activation are not involved in hydrogen bonding during base pairing, thus maintaining hybridization ability in the final conjugate.



N–Bromosuccinimide
MW 177.99

Optimal bromination of a DNA probe is in the range of 30–35 bases per 1,000 bases, a level which can be controlled by the amount of *N*-bromosuccinimide added. Over labeling can prevent specific interactions with target DNA, even if the point of initial modification is not a hydrogen bonding site.

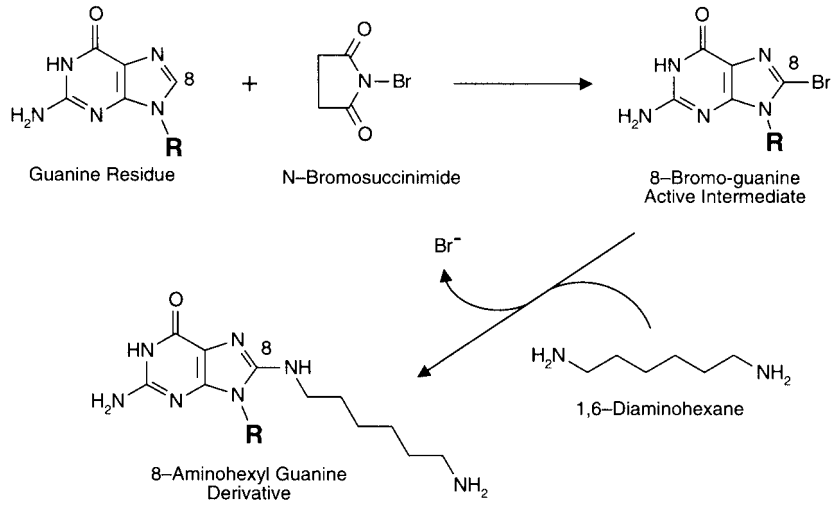


Figure 27.4 Reaction of guanine bases with *N*-bromosuccinimide causes bromination at the C-8 position of the ring. Amine nucleophiles can be coupled to this active derivative by nucleophilic displacement. Reaction of diamine compounds results in amine-terminal spacers that can be further modified to contain detectable components.

The major disadvantage with bromination is the extreme toxicity of bromine. Use a fume hood for all operations. Avoid the breathing of fumes or contact with skin or eyes. Protective clothing and gloves are recommended.

Protocol for Labeling Nucleic Acids by *N*-Bromosuccinimide Activation

Bromination

1. Mix in a microfuge tube, 20 µg of the DNA probe to be labeled, 20 µl of 1 M sodium bicarbonate, pH 9.6, and 196 µl of water. Chill on ice.
2. In a fume hood, dissolve *N*-bromosuccinimide (Thermo Fisher) in water at a concentration of 1.42 mg/ml.
3. Add 4 µl of the *N*-bromosuccinimide solution to the DNA solution (makes an 8 mM final concentration of brominating reagent). Mix well.
4. React on ice for 10 minutes. Use the bromine-activated DNA immediately.

Coupling a Diamine-Containing Spacer or Probe

1. Dissolve a diamine spacer (i.e., ethylenediamine or 1,6-diaminohexane—Thermo Fisher) in water at a concentration of 80–100 mM. *Caution*: Amine-containing molecules such as diamines are highly corrosive if they are in the free-base form (not the dihydrochlorides). Wear gloves and other protective clothing. The pH of an aqueous solution of free-base diamine will be pH > 12 and may fume. The solution also may generate heat upon dissolution of the amine. Keeping it in an ice bath will help maintain a cool solution with less fuming. Using a dihydrochloride form of a diamine, if available, will avoid the problems associated with corrosiveness, heat, and fuming.
2. Add 25 µl of the diamine solution to the bromine-activated DNA solution prepared in the Bromination section, above.
3. React for 1 hour at 50°C.
4. The diamine-modified DNA may be isolated from excess reactants by ethanol precipitation according to steps 4–7 of the protocol described previously for nick translation (Section 1, this chapter). Alternatively, dialysis or gel filtration may be done to remove excess reactants.

Conjugation via Carbodiimide Reaction with the 5′ Phosphate of DNA (Phosphoramidate Formation)

The water-soluble carbodiimide EDC (Chapter 3, Section 1.1) rapidly reacts with carboxylates or phosphates to form an active complex able to couple with amine-containing compounds. The carbodiimide activates an alkyl phosphate group to a highly reactive phosphodiester intermediate. Diamine spacer molecules or amine-containing probes then may react with this active species to form a stable phosphoramidate bond. Alternatively, bis-hydrazide compounds (Chapter 4, Section 8) may be coupled to DNA using this protocol to result in terminal hydrazide groups able to react with aldehyde-containing molecules Ghosh *et al.*, 1989). Specific labeling of DNA probes only at the 5′ end is possible using these techniques.

Carbodiimide modification of the phosphomonoester end groups on DNA molecules was first used in Khorana's lab to determine nucleotide sequences (Ralph *et al.*, 1962). That early

work used the water-insoluble reagent *N*,*N*′-dicyclohexylcarbodiimide (DCC) (Chapter 3, Section 1.4) in an organic/aqueous solvent system to effect the conjugations.

Chu *et al.* (1983, 1986) and Ghosh *et al.* (1990) describe modified carbodiimide protocols using the water-soluble reagent, EDC, instead of DCC. They also incorporate a second reactive intermediate, a phosphorimidazolide, created from the reaction of the phosphomonoester at the 5′-terminus of DNA with EDC in the presence of imidazole. A reactive phosphorimidazolide will rapidly couple to amine-containing molecules to form a phosphoramidate linkage (Figure 27.5). The chemistry had been used previously to effect the formation of phosphodiester linkages between short DNA strands (Shabarova *et al.*, 1983).

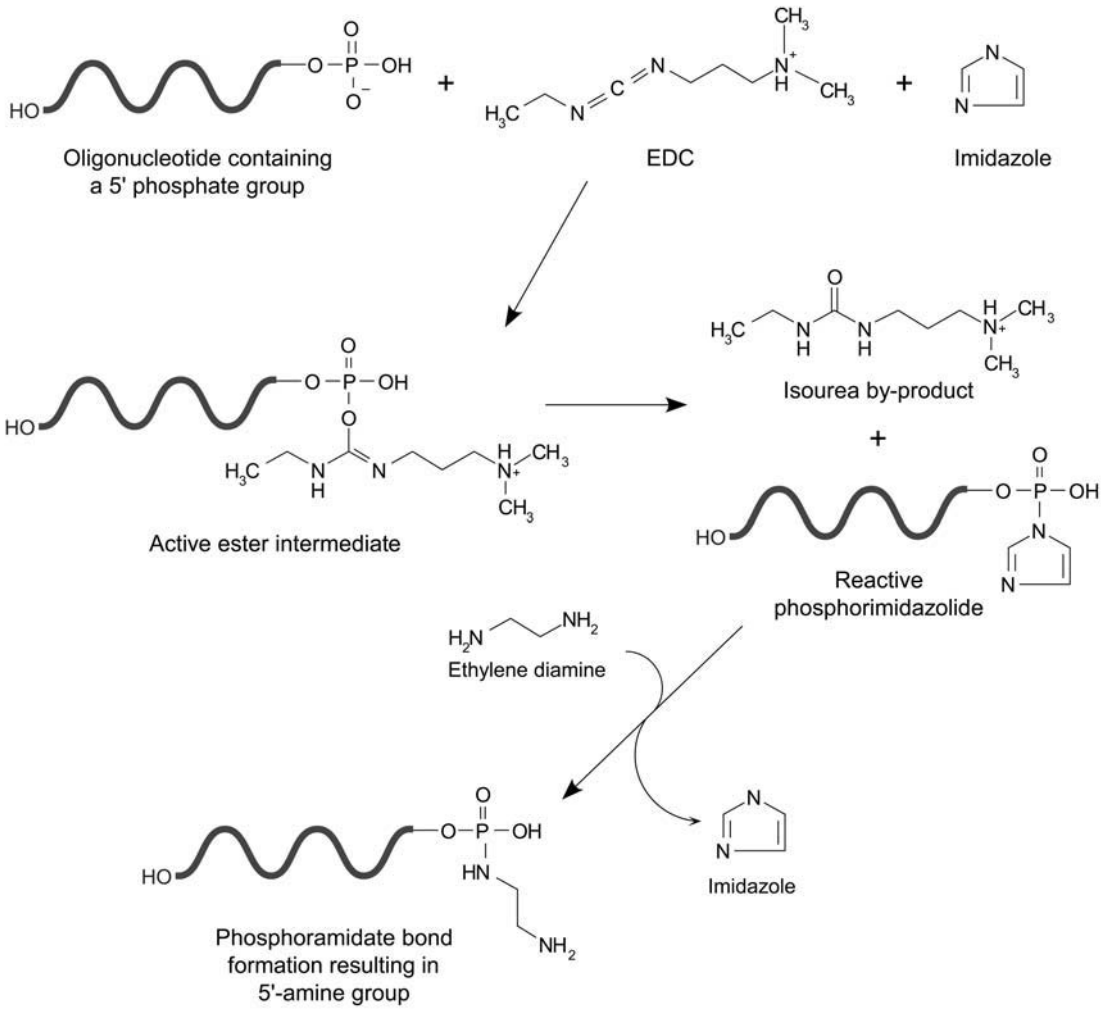


Figure 27.5 Oligonucleotides containing a 5′-phosphate group can be reacted with EDC in the presence of imidazole to form an active phosphorimidazolide intermediate. This derivative is highly reactive with amine nucleophiles, forming a phosphoramidate linkage. Diamines reacted with the phosphorimidazolide result in amine-terminal spacers that can be modified with detectable components.

The formation of a phosphorimidazolide intermediate provides better reactivity toward amine nucleophiles than the EDC phosphodiester intermediate if EDC is used without added imidazole. The EDC phosphodiester intermediate also has a shorter half-life in aqueous conditions due to hydrolysis than the phosphorimidazolide. Although EDC alone will create nucleotide phosphoramidate conjugates with amine-containing molecules (Shabarova, 1988), the result of forming the secondary phosphorimidazolide-activated species is increased derivatization yield over carbodiimide-only reactions.

The downside of EDC conjugation with oligonucleotides is the potential for reaction of the carbodiimide at the guanosine N-1 site or with thymidine residues (von der Haar *et al.*, 1971). In practice, however, this cross-reactivity appears to be low enough to maintain complete biological activity and hybridization efficiency in the final conjugate, indicating most of the derivatization occurs at the 5′ phosphate group (Chu *et al.*, 1983).

The following protocol describes the modification of DNA or RNA probes at their 5′-phosphate ends with a bis-hydrazide compound, such as adipic acid dihydrazide or carbohydrazide. A similar procedure for coupling the diamine compound cystamine can be found in Section 2.2 (this chapter).

Protocol

1. Weigh out 1.25 mg of the carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodii mide hydrochloride; Thermo Fisher) into a microfuge tube.
2. Add to the tube 7.5 µl of RNA or DNA containing a 5′ phosphate group. The concentration of the oligonucleotide should be 7.5–15 nmol or total of about 57–115.5 µg. Also immediately add 5 µl of 0.25 M bis-hydrazide compound dissolved in 0.1 M imidazole, pH 6.0. Because EDC is labile in aqueous solutions, the addition of the oligo and bis-hydrazide/imidazole solutions should be done quickly.
3. Mix by vortexing, then place the tube in a microcentrifuge and spin for 5 minutes at maximal rpm.
4. Add an additional 20 µl of 0.1 M imidazole, pH 6.0. Mix and react for 30 minutes at room temperature.
6. Purify the hydrazide-labeled oligo by gel filtration on desalting resin using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. The probe now may be used to conjugate with an aldehyde-containing molecule.

2.2. Sulfhydryl Modification of DNA

Creating a sulfhydryl group on nucleic acid probes allows conjugation reactions to be done with sulfhydryl-reactive heterobifunctional crosslinkers (Chapter 5), providing increased control over the derivatization process. Proteins can be activated with a crosslinking agent containing an amine-reactive and a sulfhydryl-reactive end, such as *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Chapter 5, Section 1.1), leaving the sulfhydryl-reactive portion free to couple with the modified DNA probe. Having a sulfhydryl group on the probe directs the coupling reaction to a discrete site on the nucleotide strand, thus better preserving hybridization ability in the final conjugate. In addition, heterobifunctional crosslinkers of this type allow two- or three-step conjugation procedures to be done, which result in better yield of the desired conjugate than when using homobifunctional reagents.

Cystamine Modification of 5′ Phosphate Groups Using EDC

DNA or RNA may be modified with cystamine at the 5′ phosphate group using a carbodiimide reaction identical to that described previously (Section 2.1, this chapter). In some procedures, the reaction is carried out in a two-step process by first forming a reactive phosphorimidazolide by EDC conjugation in an imidazole buffer. Next, cystamine is reacted with the activated oligonucleotide, causing the imidazole to be replaced by the amine and creating a phosphoramidate linkage (Chu *et al*., 1986). An easier protocol was described by Ghosh *et al*. (1990) in which the oligo, cystamine, and EDC were all reacted together in an imidazole buffer. A modification of this method developed by Zanocco *et al*. (1993) is described below.

Reduction of the cystamine-labeled oligo using a disulfide reducing agent releases 2-mercaptoethylamine and creates a thiol group for conjugation (Figure 27.6). DNA probes labeled in this manner have been successfully coupled with SPDP-activated alkaline phosphatase (Chapter 26, Sections 1.2 and 2.5), maleimide-activated horseradish peroxidase (HRP) (Chapter 26, Section 1.1), NHS-LC-biotin (Chapter 11, Section 1 and Chapter 27, Section 2.3), and the fluorescent tag AMCA–HPDP (Chapter 9, Section 3 and Chapter 27, Section 2.5).

A kit designed specifically to perform 5′-phosphate labeling on DNA probes is available from Thermo Fisher.

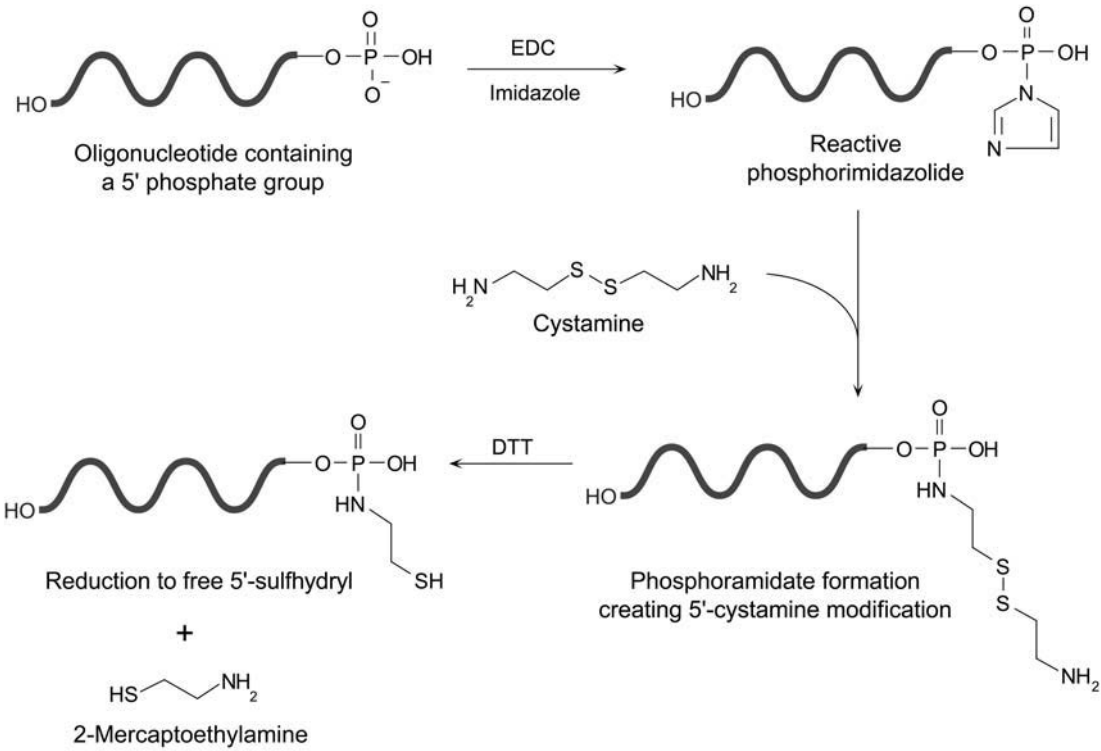


Figure 27.6 The 5′-phosphate group of oligonucleotides may be labeled with cystamine using the EDC/imidazole reaction. This results in the formation of an amine-terminal spacer containing an internal disulfide group. Reduction of the disulfide provides a route to creating a free thiol for further derivatization.

Protocol

1. Weigh out 1.25 mg of the carbodiimide EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Thermo Fisher) into a microfuge tube.
2. Add to the tube 7.5 µl of RNA or DNA containing a 5′ phosphate group. The concentration of the oligonucleotide should be 7.5–15 nmol/µl or total of about 57–115.5 µg. Also immediately add 5 µl of 0.25 M cystamine in 0.1 M imidazole, pH 6.0. Because EDC is labile in aqueous solutions, the addition of the oligo and cystamine/imidazole solutions should be done quickly.
3. Mix by vortexing, then place the tube in a microcentrifuge and spin for 5 minutes at maximal rpm.
4. Add an additional 20 µl of 0.1 M imidazole, pH 6.0. Mix and react for 30 minutes at room temperature.
5. For reduction of the cystamine disulfides, add 20 µl of 1.0 M DTT and incubate at room temperature for 15 minutes. This will release 2-mercaptoethylamine from the cystamine modification site and create the free sulfhydryl on the 5′ terminus of the oligonucleotide.
6. Purify the SH-labeled oligo by gel filtration on a desalting resin using 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. The probe now may be used to conjugate with an activated enzyme, biotin, fluorescent tag, or other molecules containing a sulfhydryl-reactive group.

SPDP Modification of Amines on Nucleotides

Oligonucleotide probes that have been modified with an amine-terminal spacer arm using any of the methods discussed in Sections 1 and 2 of this chapter may be thiolated to contain a sulfhydryl residue. Theoretically, any of the amine-reactive thiolation reagents described in Chapter 1, Section 4.1 may be used to convert an amino group on a DNA molecule into a thiol. One of the more common choices, both for crosslinking and for thiolation reactions, is the heterobifunctional reagent, SPDP (Chapter 5, Section 1.1). The NHS ester end of SPDP reacts with primary amine groups to produce stable amide bonds. The other end of the crosslinker contains a thiol-reactive pyridyl disulfide group that also can be reduced with DTT to create a free sulfhydryl.

The reaction of a 5′-diamine-modified oligonucleotide probe with SPDP proceeds under mildly alkaline conditions (optimal pH 7–9) to give the pyridyl disulfide-activated intermediate (Figure 27.7). This derivative has dual functionality. It can be used to couple directly with sulfhydryl-containing detection reagents or enzymes, or it may be converted into a free sulfhydryl for coupling to thiol-reactive compounds (Gaur *et al*., 1989; Gaur, 1991). In an alternative approach, Chu and Orgel (1988) used 2,2′-dipyridyldisulfide (Chapter 1, Section 5.2) to create reactive pyridyl disulfide groups on a reduced 5′-cystamine-labeled oligonucleotide probe. This derivative then can be used to couple with sulfhydryl-containing molecules, forming a disulfide bond.

Reduction of the pyridyl disulfide end after SPDP modification releases the pyridine-2-thione leaving group and generates a terminal—SH group. This procedure allows sulfhydryl-reactive derivatives such as maleimide-activated enzymes (Chapter 26, Section 2.3) to be conjugated with DNA probes for use in hybridization assays (Malcolm and Nicolas, 1984).

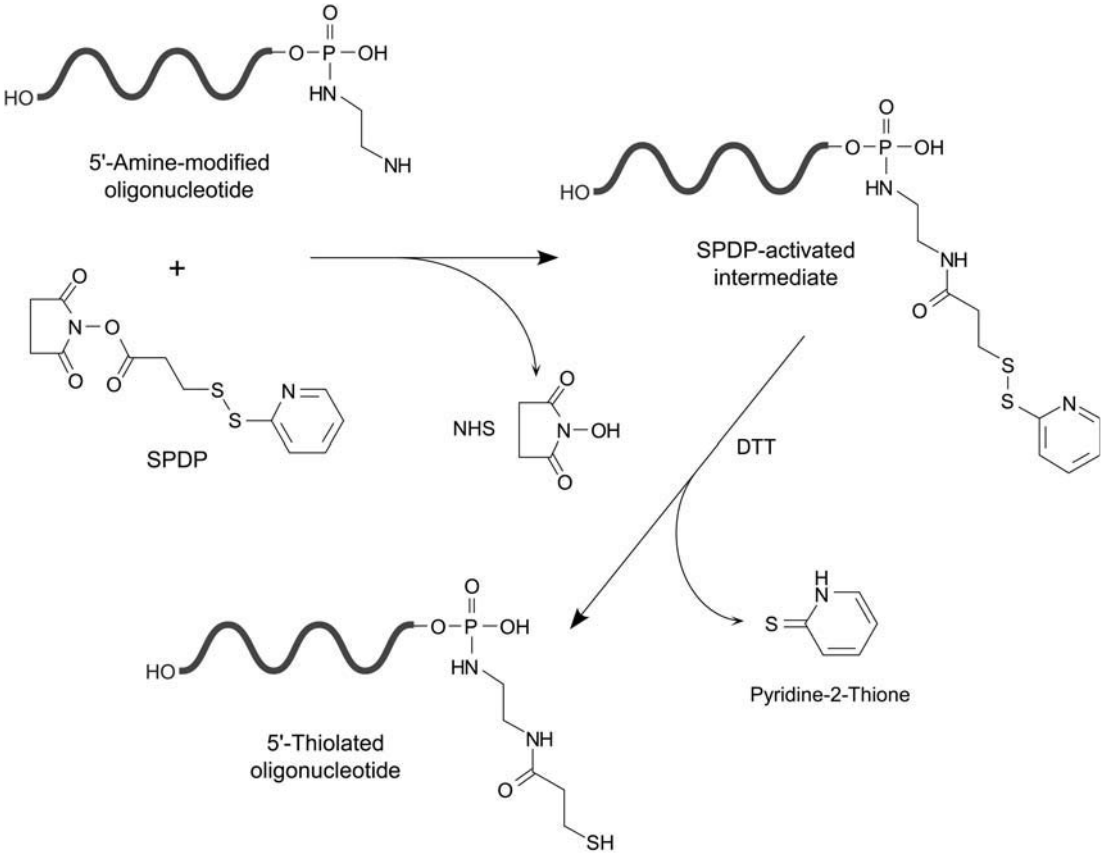


Figure 27.7 A oligonucleotide modified at its 5′-phosphate with a diamine compound may be reacted with SPDP and subsequently reduced to create a free sulfhydryl.

Protocol

1. Dissolve the amine-modified oligonucleotide to be thiolated in 250 µl of 50 mM sodium phosphate, pH 7.5.
2. Dissolve SPDP (Thermo Fisher) at a concentration of 6.2 mg/ml in DMSO (makes a 20 mM stock solution). Alternatively, LC-SPDP may be used and dissolved at a concentration of 8.5 mg/ml in DMSO (also makes a 20 mM solution). The "LC" form of the crosslinker provides a longer spacer arm that often results in better probe activity after modification. If the water-soluble sulfo-LC-SPDP is used, a stock solution in water may be prepared just prior to adding an aliquot to the thiolation reaction. In this case, prepare a 10 mM solution of sulfo-LC-SPDP by dissolving 5.2 mg/ml in water. Since an aqueous solution of the crosslinker will degrade by hydrolysis of the sulfo-NHS ester, it should be used quickly to prevent significant loss of activity.

3. Add 50 µl of the SPDP (or LC-SPDP) solution to the oligo solution. Add 100 µl of the sulfo-LC-SPDP solution, if the water-soluble crosslinker is used. Mix.
4. React for 1 hour at room temperature.
5. Remove excess reagents from the modified oligo by gel filtration on a desalting resin. The modified probe now may be used to conjugate with a sulfhydryl-containing molecule, or it may be reduced to create a thiol for conjugation with sulfhydryl-reactive molecules.
6. To release the pyridine-2-thione leaving group and form the free sulfhydryl, add 20 µl of 1.0 M DTT and incubate at room temperature for 15 minutes. If present in sufficient quantity, the release of pyridine-2-thione can be followed by its characteristic absorbance at 343 nm ($\varepsilon = 8.08 \times 10^3 M^{-1} cm^{-1}$). For many oligonucleotide modification applications, however, the leaving group will be present in too low a concentration to be detectable.
7. Purify the thiolated oligonucleotide from excess DTT by dialysis or gel filtration using 50 mM sodium phosphate, 1 mM EDTA, pH 7.2. The modified probe should be used immediately in a conjugation reaction to prevent sulfhydryl oxidation and formation of disulfide crosslinks.

SATA Modification of Amines on Nucleotides

Oligonucleotides containing amine groups introduced by enzymatic or chemical means may be modified with *N*-succinimidyl S-acetylthioacetate (SATA) (Chapter 1, Section 4.1), to produce protected sulfhydryl derivatives. The NHS ester end of SATA reacts with a primary amine to form a stable amide bond. After modification, the acetyl protecting group can be removed as needed by treatment with hydroxylamine under mildly alkaline conditions (Figure 27.8). The result is terminal sulfhydryl groups that can be used for subsequent labeling with thiol-reactive probes or activated-enzyme derivatives (Kumar and Malhotra, 1992).

The advantage of using SATA over disulfide-containing thiolation reagents such as SPDP (previous section) is that the introduction of sulfhydryl residues does not include the use of a disulfide reducing agent. Typically, the pyridyl dithiol group resulting from an SPDP thiolation must be reduced with a sulfhydryl-containing disulfide reducing compound like DTT to free the—SH group. With SATA, the sulfhydryl is freed by hydroxylamine cleavage, thus eliminating the need for removal of sulfhydryl reductants prior to a conjugation reaction.

Protocol

1. Dissolve the amine-modified oligonucleotide to be thiolated in 250 µl of 50 mM sodium phosphate, pH 8.0.
2. Dissolve SATA in DMF at a concentration of 8 mg/ml.
3. Add 250 µl of the SATA solution to the oligo solution. Mix.
4. React for 3 hours at 37°C.
5. Remove excess reagents from the modified oligo by gel filtration.
6. To deprotect the acetylated thiol group, add 100 µl of 50 mM hydroxylamine hydrochloride, 2.5 mM EDTA, pH 7.5.
7. React for 2 hours.
8. The sulfhydryl-containing oligonucleotide may be used immediately to conjugate with a sulfhydryl-reactive label, or it can be purified from excess hydroxylamine by gel filtration.

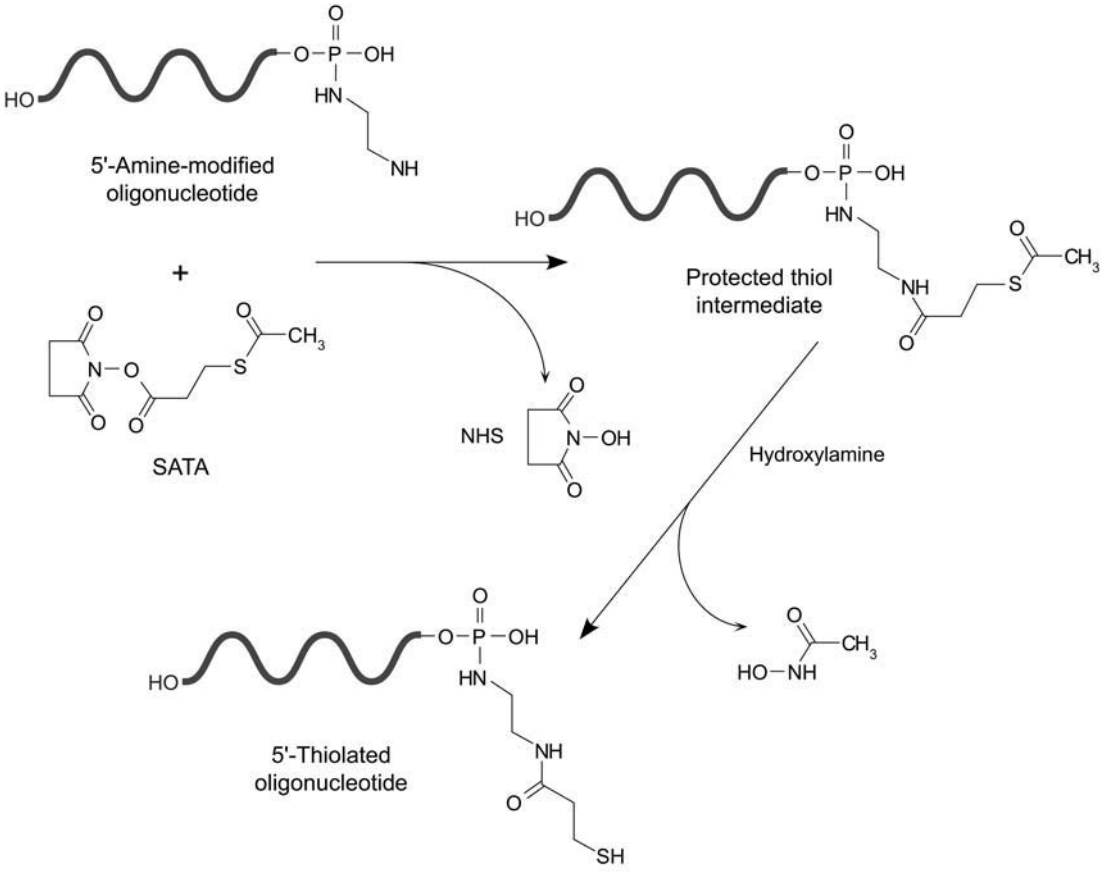


Figure 27.8 SATA may be used to modify a 5′-amine derivative of an oligonucleotide, forming a protected sulfhydryl. Deprotection with hydroxylamine results in generation of a free thiol.

2.3. Biotin Labeling of DNA

Biotinylation of oligonucleotide probes provides a highly specific biological recognition site for detection of DNA using (strept)avidin conjugates. The preparation of biotin-labeled DNA can be done either by enzymatic or chemical means. Enzyme-catalyzed reactions utilize biotinylated nucleoside triphosphates that can be incorporated into an oligonucleotide randomly or at the 3′ terminus (Section 1, this chapter). Chemical derivatization methods make use of certain reactive biotin compounds that can couple to functionally modified probes or react with DNA with the use of an activating reagent. For a description of the wide range of biotinylation compounds available, see Chapter 11. The preparation and use of avidin and streptavidin conjugates is discussed in Chapter 23.

Biotin-LC-dUTP

Perhaps the most common method of DNA biotinylation is through enzymatic incorporation with the use of a biotin-labeled deoxynucleoside triphosphate. First reported by Langer *et al*. in

1981, the procedure is probably the most popular nonradioactive labeling technique reported for oligonucleotide probes. Although biotinylated derivatives of dCTP and dATP are reported in the literature, by far the most frequently employed derivative is biotin–dUTP prepared from the reaction of an amine-modified dUTP with an amine-reactive biotinylation reagent, such as NHS-LC-biotin (Chapter 11, Section 3.1).

Biotin–dUTP derivatives are formed by modification of the C-5 position of uridine. This location is not involved in hydrogen bonding activity with complementary DNA strands, thus hybridization efficiency is not immediately compromised. By contrast, biotin–dCTP or biotin–dATP derivatives involve modification of the bases at the N-4 position of cytosine and the N-6 position of adenine, locations directly involved in hydrogen bond formation with complementary bases. Thus, DNA biotinylation through the use of modified deoxynucleoside triphosphates to be incorporated into existing DNA strands may result in better activity of the probe if dUTP is used over dATP or dCTP.

The length of the spacer arm between the C-5 position of uridine and the biotin group is another important parameter for activity of the resulting conjugate. The spacer affects the incorporation efficiency into existing probes using DNA polymerases, and it also affects the ability of an (strept)avidin conjugate to effectively bind the biotinylated probe. Designation of spacer length is usually expressed as the number of atoms separating the nucleotide base from the biotin component. Thus, biotin-*n*-dUTP would describe a biotinylated deoxynucleoside triphosphate having a spacer arm *n* atoms long. The shorter the spacer arm, the better the derivative is able to be recognized and incorporated into DNA polymers using polymerase enzymes. Conversely, the longer the spacer arm, the better the biotinylated probe is able to hybridize to its target and still maintain the capacity to have a streptavidin conjugate be complexed with it. Thus, there is an optimal trade-off in spacer length between enzymatic incorporation efficiency and labeled-probe detectability. Studies have determined that this optimal range is rather broad—between 7 atoms and 21 atoms in length. Perhaps the most common derivative is biotin-11-dUTP, wherein an 11-atom spacer is employed (Figure 27.9).

General protocols for the enzymatic incorporation of biotin-11-dUTP into DNA probes can be found in Section 1 (this chapter). A particularly interesting modification of the typical enzymatic

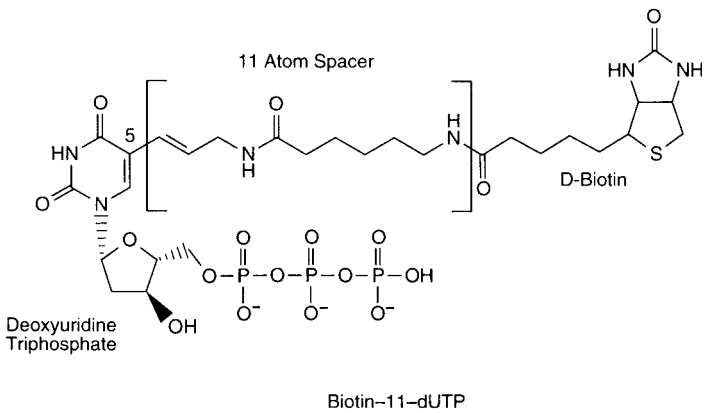


Figure 27.9 Biotin-11-dUTP is perhaps the most popular nucleotide derivative used for enzymatic biotinylation of oligonucleotides. The "11" designation refers to the number of atoms in its spacer arm.

incorporation protocol for biotin is described by Didenko (1993). The single strand template is immobilized by adsorption onto membranes before synthesis of the biotinylated probe. After polymerase incorporation of biotin-11-dUTP, the labeled probe is removed by brief heating to 90°C in water. The result is highly pure probe with no contaminating complementary DNA strands.

Photobiotin Modification of DNA

The photoreactive biotin derivative, *N*-(4-azido-2-nitrophenyl)-aminopropyl-*N*′-(*N*-d-biotinyl-3-aminopropyl)-N′-methyl-1,3-propanediamine, simply called photoactivatable biotin or photobiotin (Forster *et al*., 1985) contains a 9-atom diamine spacer group on the biotin valeric acid side chain on one end, while the other end of the spacer terminates in an phenyl azide group. The phenyl azide group can be photolyzed with UV light (350 nm) resulting in the formation of a highly reactive nitrene intermediate. In most instances, this nitrene rapidly reacts via ring expansion to form a dehydroazepine that is reactive with nucleophiles, such as amine groups (Chapter 5, Section 3).

When photobiotin is irradiated in the presence of DNA the reaction process nonselectively couples a biotin label to every 100–200 base residues. The result is an oligonucleotide probe detectable by the use of (strept)avidin conjugates. The uses of photobiotin for DNA or RNA modification are summarized in Chapter 11, Section 4.

The following protocol is based on the method of Forster *et al*. (1985). Some optimization may be necessary to obtain the best signal and activity for particular probes in hybridization assays.

Protocol for Labeling DNA Probes with Photobiotin

1. In subdued lighting conditions, dissolve photobiotin in water at a concentration of 1 μg/μ1. Protect from light.
2. Dissolve the oligonucleotide probe in water or 0.1 mM EDTA, pH 7.0, at a concentration of 1 μg/μl.
3. Mix an equal volume of the photobiotin solution with the DNA probe solution.
4. Place the solution in an ice bath and irradiate from above (about 10 cm away) for 15 minutes using a sunlamp (such as Philips Ultrapnil MLU 300 W, General Electric sunlamp RSM 275 W, or National Self-Ballasted BHRF 240–250 V 250 W W-P lamp).
5. Add 50 μl of 0.1 M Tris, pH 9, and increase the total volume of the solution to 100 μl (if it is less than this amount).
6. To extract excess photobiotin, add 100 μl of 2-butanol. Mix well and centrifuge. Discard the upper phase. Repeat this process two more times.
7. To recover the biotinylated DNA, add 75 μl of 4 M NaCl and mix.
8. Add 100 μl of ethanol and cool the sample in dry ice (CO_2) for 15 minutes.
9. Centrifuge to collect the precipitated, biotinylated DNA.

Reaction of NHS-LC-Biotin with Diamine-Modified DNA Probes

NHS-LC-biotin is an extended spacer arm derivative of biotin containing an amine-reactive NHS ester (Chapter 11, Section 1). The compound is a popular choice for biotinylating a wide

range of molecules containing primary amine groups, especially proteins. Oligonucleotides modified to contain amine-terminal spacer arms also can be modified with NHS-LC-biotin to create stable amide bond derivatives. Alternatively, a hydrophilic biotinylation compound containing a PEG spacer arm can be used to form a biotin-oligo derivative without the hydrophobic character of the alkyl chain within NHS-LC-biotin. Chapter 18, Section 3 describes the amine-reactive NHS–PEG_n–biotin compounds suitable for this purpose.

Whether an amine is incorporated into an oligo by enzymatic means or chemical derivatization, an NHS-ester-containing biotinylation reagent can be used to label the derivative in high yield. If an amine group is added to the 5′ end of a DNA probe by phosphoramidate formation (Section 2.1, this chapter), then biotinylation of such molecules directs the label to a region totally removed from interfering in subsequent hybridization with a target DNA strand (Figure 27.10).

The following protocol assumes that the amine-containing oligo has already been synthesized by any of the methods discussed in Section 2.1, this chapter.

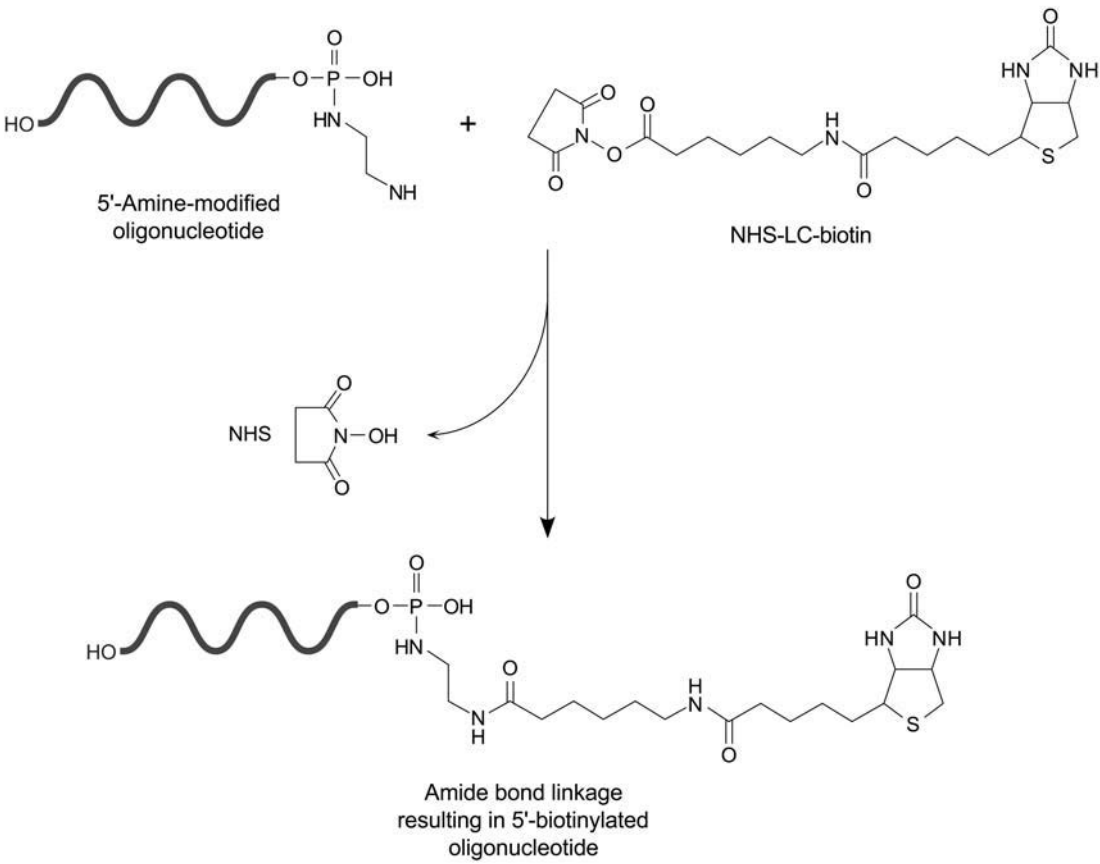


Figure 27.10 Biotinylation of oligonucleotides may be done at the 5′-phosphate end using a diamine derivative and reacting with NHS-LC-biotin.

Protocol

1. Prepare 10–20 μg of amine-containing oligonucleotide in 200 μl of water. Add to this solution, 20 μl of 1 M sodium bicarbonate, pH 9.0.
2. Dissolve NHS-LC-biotin (Thermo Fisher) in DMSO at a concentration of 10 mg/ml. Add 50 μl of the biotinylation solution to the oligo solution. Mix well.
3. React for 2 hours at room temperature.
4. Isolate the biotinylated probe by ethanol/salt precipitation as described in Section 1 (this chapter) for nick-translation modification of DNA probes. Alternatively, dialysis, gel filtration, or *n*-butanol extraction may be used to remove excess reagents.

Biotin–Diazonium Modification of DNA

Diazonium groups are able to couple at the C-8 position of adenosine or guanosine residues, forming diazo bonds. *ρ*-Aminobenzoyl biocytin can be used in this reaction to add a biotin handle to purine bases within oligonucleotides (Chapter 11, Section 5). This biotinylation reagent contains a 4-aminobenzoic acid amide derivative off the α-amino group of biocytin's lysine residue (Thermo Fisher). The aromatic amine can be treated with sodium nitrite in dilute HCl to form a highly reactive diazonium derivative, which is able to couple with active hydrogen-containing compounds. A diazonium reacts rapidly with histidine or tyrosine residues within proteins, forming covalent diazo bonds (Wilchek *et al.*, 1986). It also can react with purine residues within DNA at position 8 of the bases (Rothenberg and Wilchek, 1988; Lowe, 1979) (Figure 27.11).

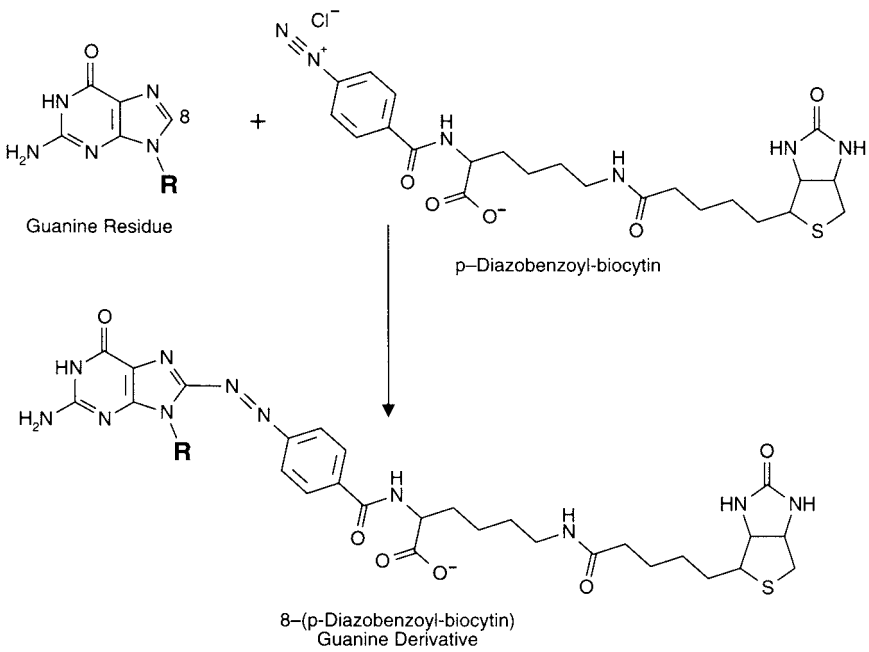


Figure 27.11 This diazo derivative of biocytin may be used to modify guanine bases at the C-8 position.

Protocol

1. Prepare diazotized ρ-aminobenzoyl biocytin by using the protocol outlined in Chapter 11, Section 5, but instead of starting with 2 mg the biotinylation reagent dissolved in 40 µl of 1 N HCl, use 9 mg in 180 µl of 1 N HCl. Proportionally scale up the other reactant quantities used in the protocol. After the reaction is complete, immediately adjust the pH of the final solution to 9.
2. Add 1 µg of single-stranded DNA to the above solution.
3. React for 30 minutes at room temperature.
4. Purify the biotinylated DNA probe by ethanol precipitation, gel filtration, *n*-butanol extraction, or dialysis as discussed in previous sections.

Reaction of Biotin–BMCC with Sulfhydryl-Modified DNA

Biotin–BMCC is a sulfhydryl-reactive biotinylation reagent containing a maleimide group at the end of an extended spacer arm. The long spacer (32.6 Å) provides enough distance between modified oligonucleotides and the bicyclic biotin end to allow efficient binding of (strept)avidin probes, even when hybridized to target sequences. The reagent may be used to add a biotin label to DNA or RNA molecules after they have been modified to contain thiol groups. For instance, cystamine labeling at the 5′ phosphate group of DNA via carbodiimide-mediated phosphoramidate formation followed by disulfide reduction (Section 2.2, this chapter) can create the required sulfhydryl groups. Subsequent reaction with biotin–BMCC results in a derivative labeled only at an end of the DNA probe (Figure 27.12), thus avoiding the potential for hydrogen bonding interference in hybridization assays.

Since maleimide groups are highly specific for coupling to thiols in the pH range of 6.5 to 7.5, side reaction products can be avoided. The reaction is complete within two hours at room temperature.

Protocol

1. Prepare 10–20 µg of a sulfhydryl-containing oligonucleotide in 200 µl of 50 mM sodium phosphate, 10 mM EDTA, pH 7.2 (the methods outlined in Section 2.2 of this chapter can be used to form the thiol group).
2. Dissolve biotin–BMCC in DMSO at a concentration of 5 mg/ml. Prepare fresh.
3. Add 50 µl of the biotinylation solution to the oligo. Mix well.
4. React for 2 hours at room temperature.
5. Isolate the biotinylated probe by ethanol/salt precipitation as described in Section 1 for nick translation (this chapter).

Biotin–Hydrazide Modification of Bisulfite-Activated Cytosine Groups

Biotin–hydrazide is the hydrazine derivative of D-biotin prepared using its valeric acid carboxylate (Chapter 11, Section 3). The hydrazide group typically is used to react with aldehyde and ketone groups to give hydrazone linkages. However, the hydrazide compound also can undergo transamination reactions with cytosine residues via catalysis with bisulfite (Section 2.1, this chapter) (Figure 27.13). DNA or RNA probes containing cytosine groups may be modified to contain biotin labels using a simple, one-step procedure (Reisfeld *et al.*, 1987). The detection limit of DNA probes biotinylated using this technique can be less than 1 pg on blots, when analyzed

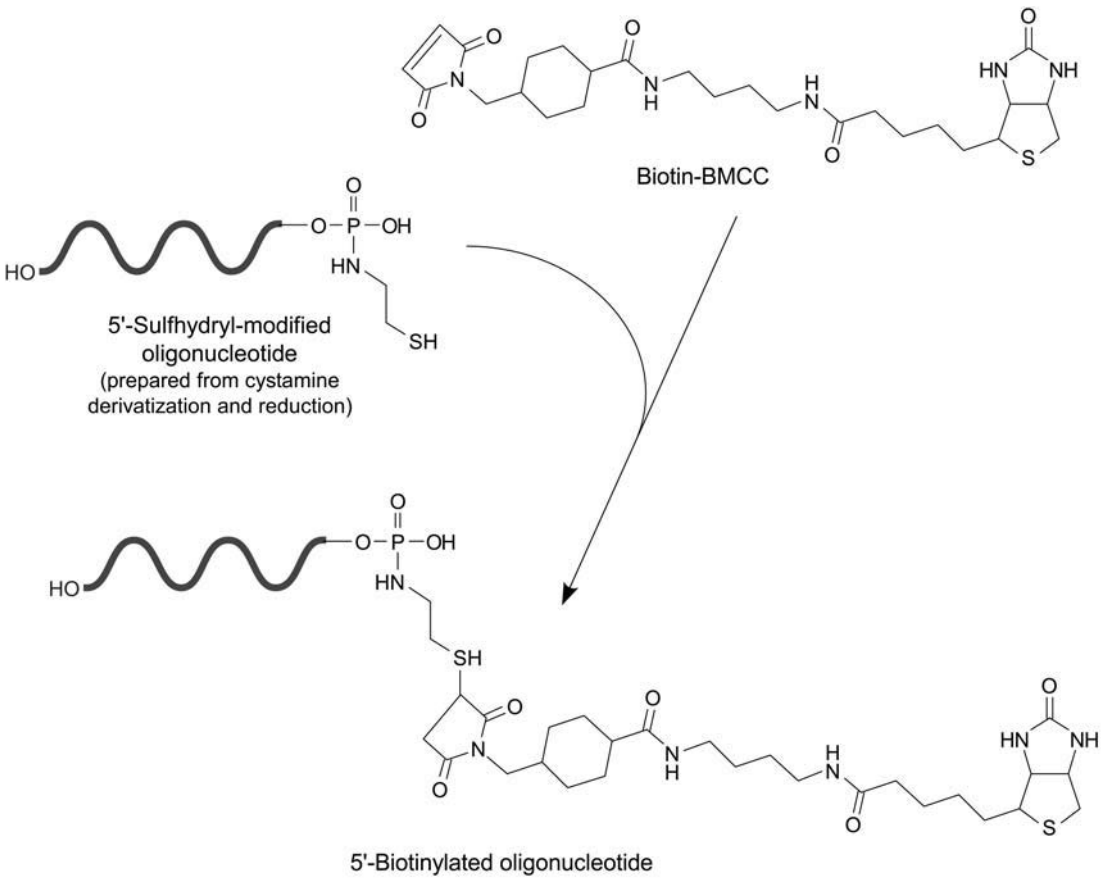


Figure 27.12 Biotin–BMCC can be used to modify a reduced, cystamine derivative of DNA, forming a thioether linkage.

using a streptavidin–HRP conjugate with chemiluminescent detection. A longer chain analog of biotin–hydrazide, biotin-LC-hydrazide, may be used to create an extended spacer between the oligonucleotide and the bicyclic biotin group, thus increasing the binding efficiency of avidin or streptavidin conjugates. Alternatively, a hydrophilic biotin–PEG–hydrazide compound may be used in this reaction to provide greater water solubility for the label (Chapter 18, Section 3). Leary *et al.* (1983) reported that increasing the spacer arm length from 4 to 11 atoms when biotinylating DNA probes can increase the detectability of the target DNA approximately 4-fold.

The following method is adapted from Reisfeld *et al.* (1987).

Protocol

1. Prepare 50 µg of a single-stranded DNA probe in 300 µl of 50 mM sodium acetate, pH 4.5.
2. Dissolve biotin–hydrazide in water at a concentration of 10 mg/ml.
3. Add 300 µl of the biotin–hydrazide solution to the DNA solution.
4. Add sodium bisulfite to obtain a final concentration of 1 M in the reaction medium.

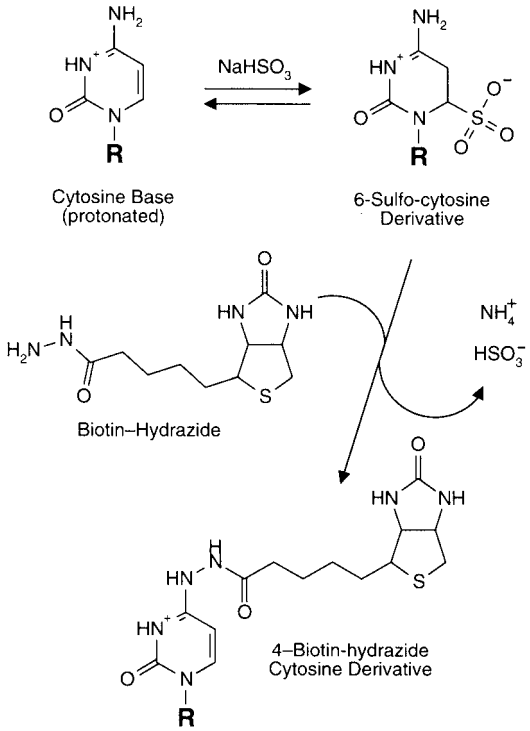


Figure 27.13 Biotin–hydrazide may be incorporated into cytosine bases using a bisulfite-catalyzed transamination reaction.

5. React for 24 hours at 37°C.
6. Remove excess reactants by dialysis against water at 4°C.

2.4. Enzyme Conjugation to DNA

Enzymes useful for detection purposes in ELISA techniques (Chapter 26) also can be employed in the creation of highly sensitive DNA probes for hybridization assays. The attached enzyme molecule provides detectability for the oligonucleotide through turnover of substrates that can produce chromogenic or fluorescent products. Enzyme-based hybridization assays are perhaps the most common method of nonradioactive detection used in nucleic acid chemistry today. The sensitivity of enzyme-labeled probes can approach or equal that of radiolabeled nucleic acids, thus eliminating the need for radioactivity in most assay systems.

The conjugation reactions involved in DNA–enzyme crosslinking are not unlike the methods used to form antibody–enzyme conjugates (Chapter 20, Section 1). Bifunctional crosslinkers can be used to couple a modified oligonucleotide to an enzyme molecule using the same basic principles effective in protein–protein conjugation. The only requirement is that the DNA molecule be modified to contain one or more suitable functional groups, such as nucleophiles like amines or sulfhydryls. The modification process used to create these functional groups can use enzymatic (Section 1, this chapter) or chemical (Section 2, this chapter) means and it can result

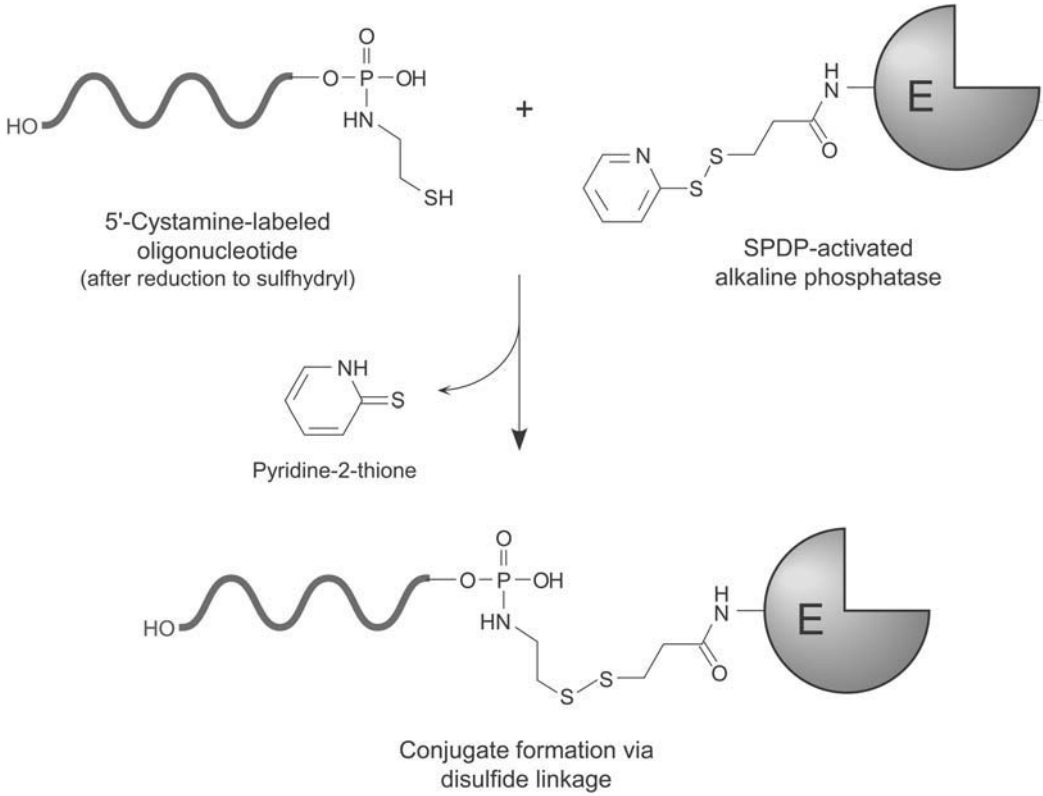


Figure 27.14 An oligonucleotide modified with cystamine and reduced to generate a free sulfhydryl may be conjugated with an SPDP-modified enzyme, forming a disulfide linkage.

in random incorporation of modification sites or be directed exclusively at one end of the DNA molecule, such as in 5′ phosphate coupling.

The following sections describe some of the more common procedures of preparing DNA–enzyme conjugates.

Alkaline Phosphatase Conjugation to Cystamine-Modified DNA Using Amine- and Sulfhydryl-Reactive Heterobifunctional Crosslinkers

A cystamine group added to the 5′ phosphate of DNA molecules using a carbodiimide reaction (Section 2.2, this chapter) can be used in a heterobifunctional crosslinking scheme to conjugate with alkaline phosphatase. Crosslinking agents containing an amine-reactive portion and a sulfhydryl-reactive part work best in forming this type of conjugate. Perhaps one of the most common heterobifunctional reagents used for DNA–enzyme formation is SPDP (Chapter 5, Section 1.1). SPDP contains an NHS ester on one end able to create an amide bond linkage with amino groups on protein molecules. After modification of alkaline phosphatase with this crosslinker, the enzyme is activated to contain pyridyl disulfide groups for coupling to the sulfhydryls on a cystamine-modified DNA probe (Figure 27.14). The reaction forms disulfide

bonds between the oligonucleotide and the alkaline phosphatase enzyme. Since the crosslink occurs only at the 5′ end of the DNA strand, the presence of an enzyme molecule does not adversely affect the ability of base pairing and hybridization to a target sequence.

The following protocol assumes that the labeling process used to create a sulfhydryl-modified DNA probe already has been done according to the method of Section 2.2 (this chapter). The modification procedure for activating alkaline phosphatase with SPDP may be done according to the protocol described in Chapter 5, Section 1.1. To obtain efficient labeling of all the alkaline phosphatase added to the reaction medium, the modified oligo is reacted in a 10-fold molar excess. Reaction of the DNA probe in excess allows easy separation of not-coupled oligo from conjugated probe, thus eliminating any potential interference in hybridization assays due to unlabeled oligonucleotide.

Protocol

1. Dissolve a 5′-sulfhydryl-modified oligonucleotide in water or 10 mM EDTA at a concentration of 0.05–25 μg/μl. Calculate the total nmoles of oligo present based upon its molecular weight.
2. Prepare SPDP-activated alkaline phosphatase in 50 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2. Add to the oligo solution, an amount of the activated enzyme representing a 10-fold molar excess over the calculated amount of DNA present.
3. React at room temperature for 30 minutes with gentle mixing.
4. The alkaline phosphatase–DNA conjugate may be purified away from excess oligo by dialysis, gel filtration, or through the use of centrifugal concentrators. A simple way of removing unreacted oligo is to use Centricon-30 concentrators (Amicon) which have a molecular weight cutoff of 30,000. Since the enzyme molecular weight is approximately 140,000 and the conjugate is even higher, a relatively small DNA probe will pass through the membranes of these units while the conjugate will not. To purify the conjugate using Centricon-30 s, add 2 ml of the phosphate buffer from step 2 to one concentrator unit, then add the reaction mixture to the buffer and mix. Centrifuge at 1,000 g for 15 minutes or until the retentate volume is about 50 μl. Add another 2 ml of buffer and centrifuge again until the retentate is 50 μl. Invert the Centricon-30 unit and centrifuge to collect the retentate in the collection tube provided by the manufacturer.

Alkaline Phosphatase Conjugation to Diamine-Modified DNA Using DSS

Disuccinimidyl suberate (DSS) is a homobifunctional crosslinker containing an amine-reactive NHS ester at both ends (Chapter 4, Section 1.2). Reaction of the reagent in excess with diamine-modified DNA probes creates an activated intermediate able to conjugate with enzyme molecules through their available amine groups (Figure 27.15) (Jablonski *et al*., 1986). The coupling reaction produces stable amide linkages under mildly alkaline conditions. Although the following protocol has been used to label oligonucleotides with success, it may be less efficient than the previous protocol at forming the desired conjugate due to the homobifunctional nature of the crosslinker. During the activation step, the modified DNA must be purified away from excess DSS. Since this is done under aqueous conditions, hydrolysis of the free NHS ester at the other end of the crosslinker takes place at the same time. Activity losses can be severe if the separation step is not done rapidly. In fact, the original protocol called for several hours

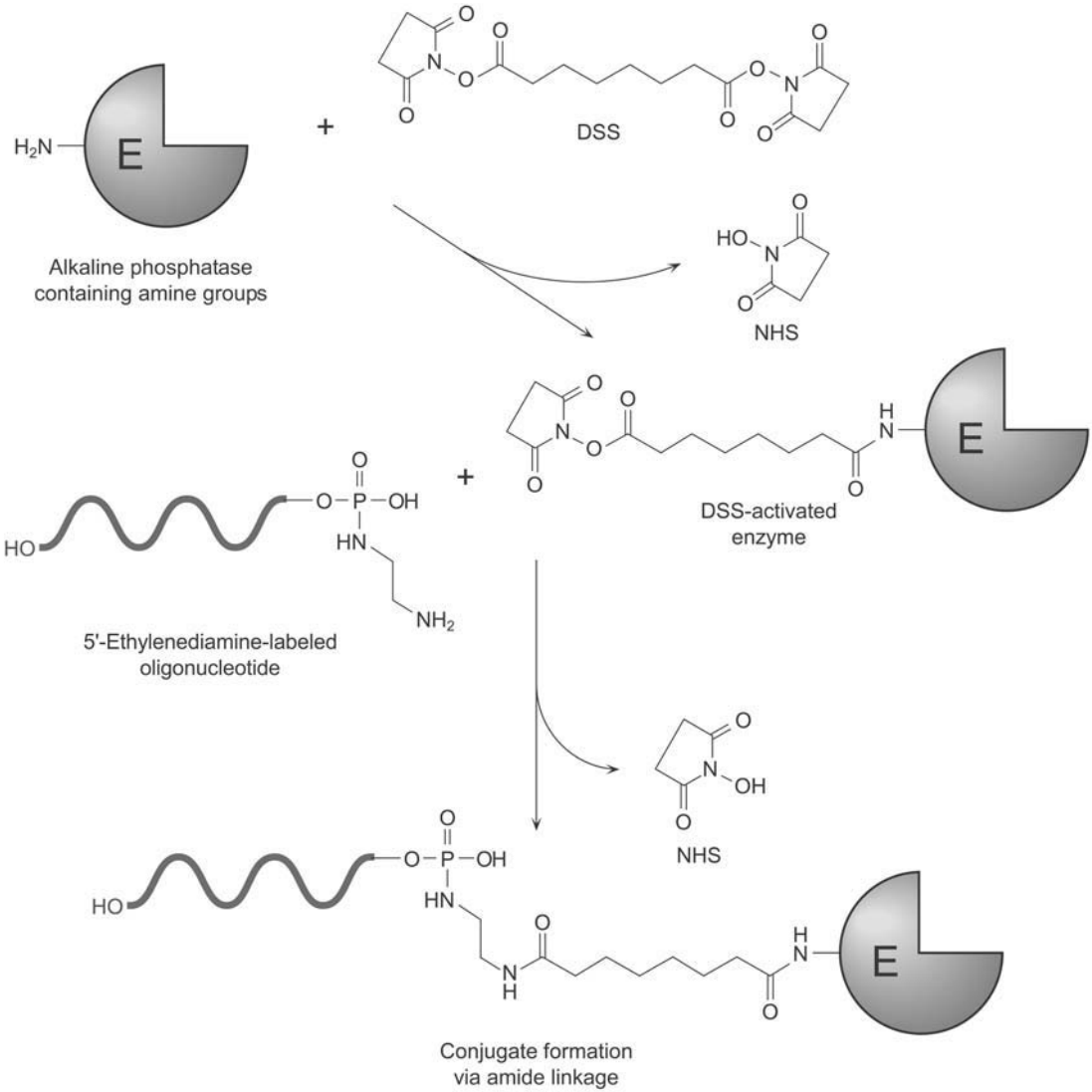


Figure 27.15 The homobifunctional crosslinker DSS may be used to conjugate an enzyme to a 5′-diamine-modified oligonucleotide. The NHS ester groups on DSS react with the amines to form amide bonds.

of gel filtration chromatography and concentration before the conjugation reaction was done. Farmer and Castaneda (1991) made a significant improvement to this procedure by including a faster separation step using alcohol extraction after activation of the oligo with DSS. The purification time decreased to minutes instead of hours. This does help to limit hydrolysis, but cannot completely avoid it.

The following protocol is based on the methods of Farmer and Castaneda (1991), Kiyama *et al.* (1992), and Ruth (1993).

Protocol

1. Prepare an amine-modified oligonucleotide according to any of the protocols discussed in Section 2.1 (this chapter). Dissolve or buffer-exchange the oligo into 0.1 M sodium borate, 2 mM EDTA, pH 8.25, at a concentration of 9 nmol (2.0 A_{260nm} units) in 15 µl.
2. Dissolve DSS in dry DMSO at a concentration of 1 mg/100 µl. Prepare fresh.
3. Add 30 µl of the DSS solution to the oligo. Mix well.
4. React for 15 minutes at room temperature in the dark.
5. Immediately extract excess DSS and reaction by-products by the addition of 0.5 ml of *n*-butanol. Mix vigorously by vortexing and centrifuge (1 minute, 15,000 rpm) to separate the two phases. Carefully remove the upper layer and discard. Extract two more times with *n*-butanol.
6. Chill the remaining sample on dry ice and lyophilize to remove the last traces of liquid. The drying period will only take 15–30 minutes. The dried, DSS-activated DNA is stable under anhydrous conditions.
7. Dissolve or dialyze alkaline phosphatase into 3 M NaCl, 30 mM triethanolamine, 1 mM MgCl2, pH 7.6, at a concentration of 20 mg/ml.
8. Add 70 µl of the alkaline phosphatase to the dried, DSS-activated DNA. Mix gently to dissolve.
9. React overnight at 4°C in the dark.
10. Remove unconjugated oligo by using a Centricon-30 centrifugal concentrator according to step 4 of the protocol described in the previous section. Unconjugated enzyme may be removed by ion-exchange chromatography using a MonoQ-10 (0.5 × 5 cm) FPLC column (GE Healthcare) or the equivalent. Binding buffer is 20 mM Tris, pH 8. Elute using a linear gradient of 0–100 percent 20 mM Tris, 1 M NaCl, pH 8. Free enzyme will elute before the more negatively charged oligo–enzyme conjugate.

Enzyme Conjugation to Diamine-Modified DNA Using PDITC

PDITC, 1,4-phenylene diisothiocyanate, is a homobifunctional crosslinker containing two amine-reactive isothiocyanate groups on a phenyl ring. Reaction in excess with an amine-modified oligonucleotide results in the formation of a thiourea linkage, leaving the second isothiocyanate group free to couple with amine-containing enzymes or other molecules (Urdea *et al.*, 1988) (Figure 27.16).

The following protocol is adapted from Keller and Manak (1989).

Protocol

1. Prepare an amine-modified oligonucleotide using any of the methods of Sections 1 or 2.1 (this chapter). Dissolve or buffer exchange 70 µg of the oligo into 25 µl of 0.1 M sodium borate, pH 9.3.
2. Dissolve 10 mg of PDITC (Aldrich) in 500 µl of DMF. Add this solution to the oligo prepared in step 1.
3. React for 2 hours at room temperature in the dark.
4. To extract excess reactant, add to the reaction medium, 3 ml of *n*-butanol and 3 ml of water. Mix well. Centrifuge the mixture to separate the two phases. Discard the upper

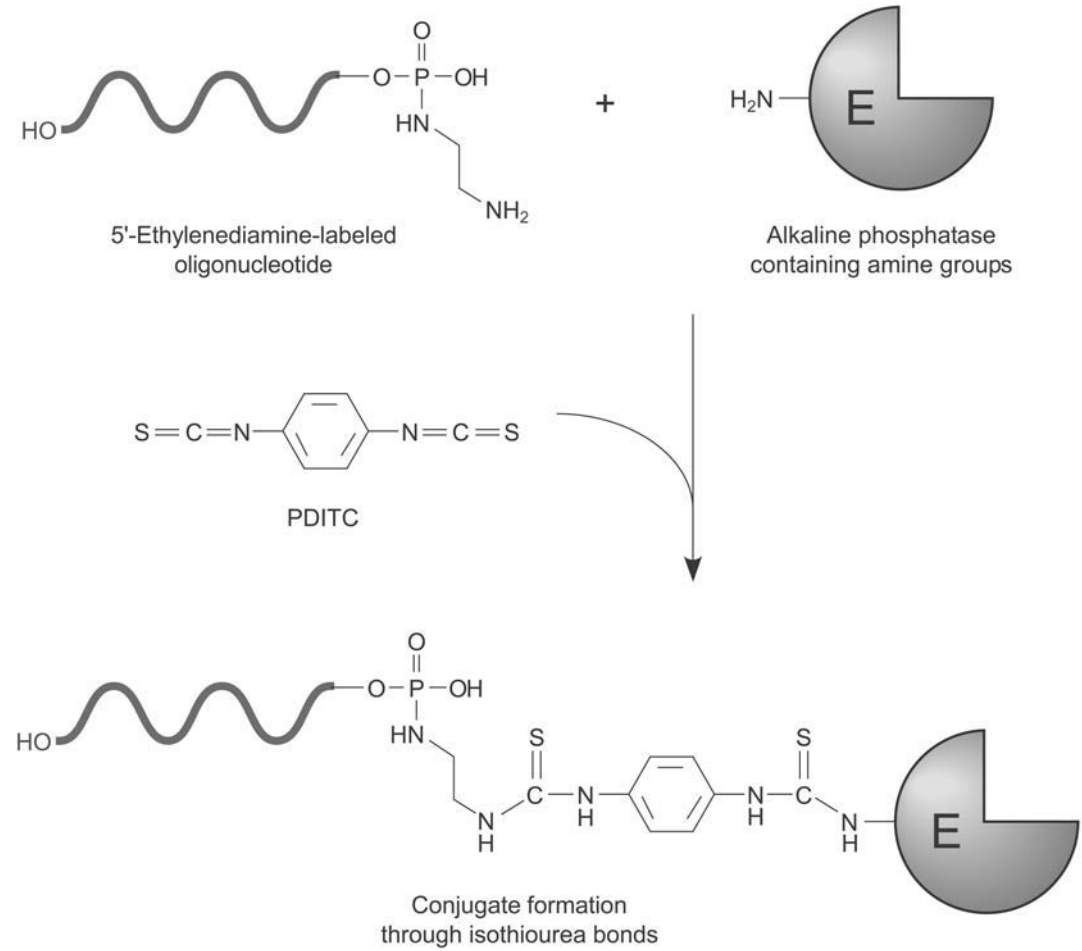


Figure 27.16 The homobifunctional crosslinker PDITC may be used to conjugate an enzyme to a 5′-diamine-modified oligonucleotide, creating isothiourea linkages.

yellow layer. Repeat the extraction process several times, and then dry the remaining solution containing activated oligo using lyophilization or a rotary evaporator. The PDITC-activated DNA is stable in a dried state.

5. Dissolve HRP (Chapter 26) in 200 µl 0.1 M sodium borate, pH 9.3, at a concentration of 10 mg/ml. If the HRP is supplied as an ammonium sulfate suspension, all ammonium ions must be removed by extensive dialysis prior to the conjugation reaction. Add the HRP solution to the activated oligo.
6. React overnight at room temperature in the dark.
7. Excess enzyme may be removed through isolation of the oligo–enzyme conjugate using electrophoresis separation under nondenaturing conditions. The reaction solution is applied to a 7 percent polyacrylamide gel using 90 mM Tris, 90 mM Boric acid, 2.7 mM EDTA, pH 8.3, as the running buffer. The conjugate appears as a brown band in the middle of the gel.

Conjugation of SFB-Modified Alkaline Phosphatase to Bis-Hydrazide-Modified Oligonucleotides

DNA probes modified to contain a 5′-terminal hydrazide group (Section 2.1, this chapter) can be conjugated to aldehyde-containing molecules, resulting in the formation of a hydrazone bond. The crosslinking agent succinimidyl *p*-formylbenzoate (SFB; Chapter 17, Section 2) can be used to add aldehyde groups to proteins and other molecules that do not naturally contain them (Chapter 1, Section 4.4). Reaction of SFB-modified alkaline phosphatase with a hydrazide–DNA derivative can produce a conjugate having excellent sensitivity for use as a hybridization probe (Figure 27.17). Other enzymes and detection molecules modified to contain aldehydes may be coupled to hydrazide–DNA probes using similar methods. Using an SFB-modified HRP (or periodate-oxidized HRP) that contains aldehyde groups to prepare the DNA conjugate gives about a 40-fold less sensitivity in hybridization assays when compared to an alkaline phosphatase conjugate in this procedure (Ghosh *et al*., 1989). However, this result may change if chemiluminescent detection using an HRP conjugate is done, as this method can result in femtogram detection limits.

The following protocol assumes the prior derivatization of an oligonucleotide at the 5′ end using a bis-hydrazide compound according to the protocol of Section 2.1 (this chapter) using a carbodiimide-mediated reaction.

Protocol

1. Dissolve alkaline phosphatase at a concentration of 10 mg/ml in 0.1 M sodium bicarbonate, 3 M NaCl, pH 8.5. Dialyze against this solution if the enzyme is already dissolved in another buffer. This protocol requires at least 0.4 ml of the enzyme solution.
2. Dissolve SFB in acetonitrile at a concentration of 50 mM (12.35 mg/ml). Make at least 100 µl.
3. Add 40 µl of the SFB solution to the 0.4 ml alkaline phosphatase solution with mixing.
4. React for 30 minutes at room temperature.
5. Remove excess reactants by dialysis against 50 mM MOPS, 0.1 M NaCl, pH 7.5.
6. Add the aldehyde-derivatized alkaline phosphatase to 8 nmol of a 5′-hydrazide oligonucleotide preparation made according to Section 2.1 (this chapter) using a carbodiimide coupling protocol.
7. React overnight at room temperature.
8. Remove unconjugated oligonucleotide using gel filtration on a column of Bio-Rad P-100 (1.5 × 65 cm). Use 50 mM Tris, pH 8.5 as the chromatography buffer. Pool the enzyme fractions and apply the sample to a 1 × 7 cm column of DEAE–cellulose equilibrated with the same buffer. After washing the column with 0.1 M Tris, pH 8.5, a salt gradient from 0 to 0.2 M NaCl in the same buffer is used to remove unconjugated enzyme. The enzyme–DNA conjugate is then eluted with 0.1 M Tris, 0.5 M NaCl, pH 8.5.

2.5. Fluorescent Labeling of DNA

Oligonucleotide probes may be labeled with small fluorescent molecules for detection of hybridization by luminescence. Fluorescent probes are widely used in assay systems involving bio-specific interactions (Chapter 9). Receptors for ligands may be localized in tissues or cells by modification of the ligand with the appropriate fluorophore. Targeted molecules may be

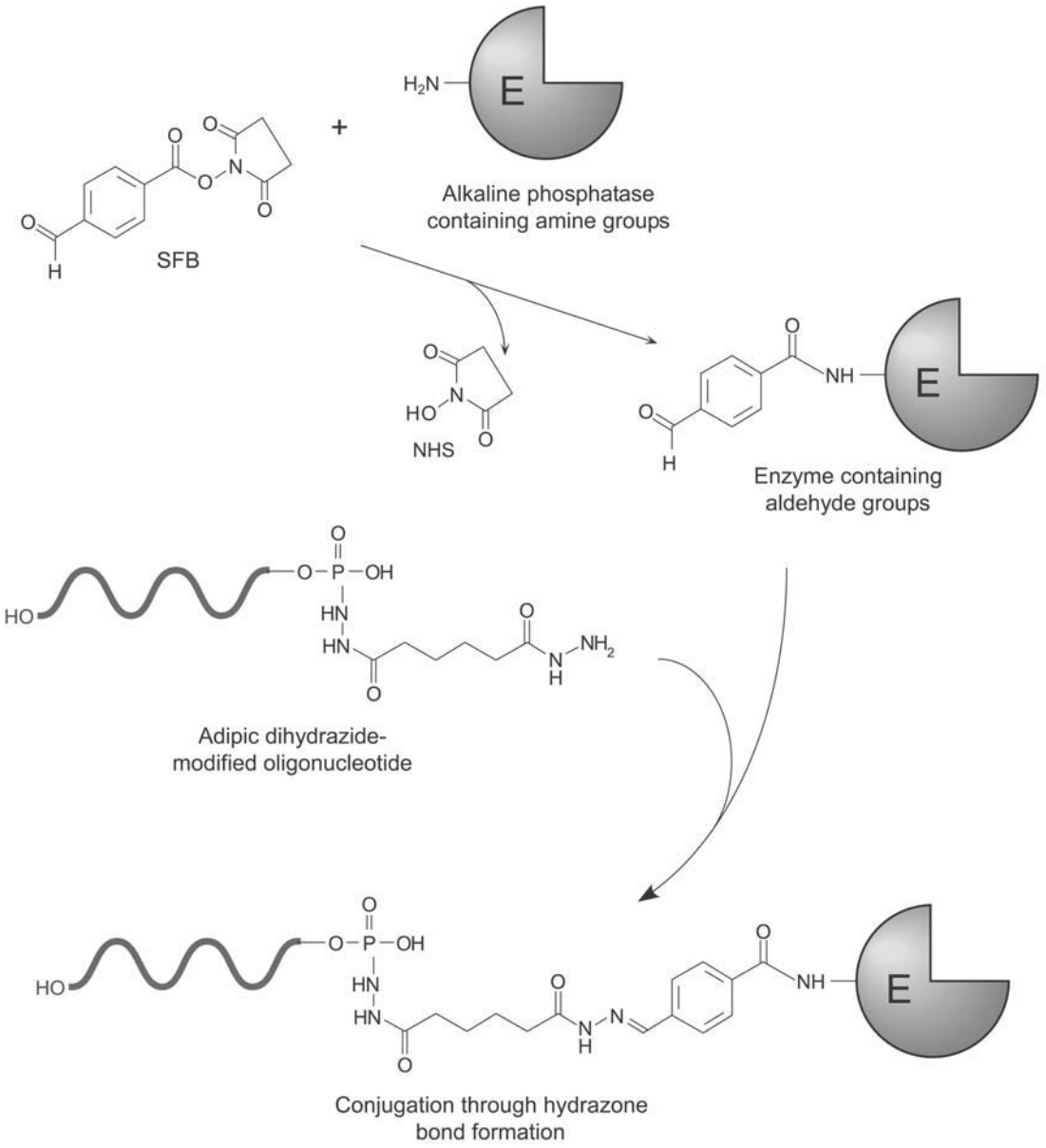


Figure 27.17 SFB may be used to create aldehyde groups on enzyme molecules for subsequent conjugation to a 5′-bis-hydrazide-modified oligonucleotide, forming hydrazone bonds.

quantified through measurement or modulation of a fluorescent signal upon binding of a tagged ligand. The sensitivity of fluorescent assays can approach that obtained using radiolabels.

Fluorescently labeled DNA probes can be used for detection, localization, or quantification of target DNA sequences. *In situ* hybridization mapping of genomic DNA sequences can be

done using fluorescent probes to target particular regions within chromosomes. Called FISH for fluorescent *in situ* hybridization, the technique is used extensively to identify marker chromosomes or chromosomal rearrangements. Since many genomic sequences are repeated, usually occurring in multiple copies within isolated regions of the chromosome, the fluorescent label on the DNA probe allows localization of targeted genes with high sensitivity. For a review of FISH, see Meyne (1993).

Fluorescently labeled DNA probes also can be used in homogeneous assay systems to detect and quantify target complementary sequences. The majority of these systems use a process of energy transfer and fluorescent quenching to detect hybridization phenomena. The principle of these assays involves the labeling of two binding components that can specifically interact with a target DNA. One or both of the labels may be a luminescent compound. The luminescent quality of the first label may consist of a chemiluminescent probe that can be excited through specific chemical processes, producing light emission. Alternatively, the label may be a fluorescent probe which can absorb light of a particular wavelength and subsequently emit light at another wavelength.

The second label also may be a fluorescent compound, but doesn't necessarily have to be. As long as the second label can absorb the emission of the first label and modulate its signal, binding events can be observed. Thus, the two labeled DNA probes interact with each other to produce fluorescence modulation only after both have bound target DNA and are in enough proximity to initiate energy transfer. Common labels utilized in such assay techniques include the chemiluminescent probe, *N*-(4-aminobutyl)-*N*-ethylisoluminol, and reactive fluorescent derivatives of fluorescein, rhodamine, and the cyanine dyes (Chapter 9). For a review of these techniques, see Morrison (1992).

To prepare labeled DNA molecules for use in fluorescent assays, the oligo must be first derivatized to contain a functional group. Any of the methods of Sections 2.1 and 2.2 (this chapter) may be used to add an amine or sulfhydryl residue to specific regions of the DNA polymer. Once modified in this manner, the oligo may be further reacted with a fluorescent probe to create the final derivative. However, many of the fluorescent quenching formats exclusively specify either 3′- or 5′-labeled DNA molecules. This is because discrete modification at just one end of the oligo assures that the label on a hybridized probe will be near enough to a second hybridized-and-labeled DNA molecule to effect the luminescent modulation necessary to make the system viable. Multiple fluorescent labels on nucleotides within the DNA probe would not be affected to the same degree by a second label attached to another oligo hybridized some distance away on the target strand. Therefore, use of the terminal transferase method of adding a modified nucleoside triphosphate to the 3′ end (Section 1) or 5′-phosphate modification using a carbodiimide-mediated reaction (Section 2.1) work best for creating functionalized DNA derivatives for fluorescent modulation techniques.

Another method of fluorescent detection of DNA or RNA targets involves the modification of a targeting oligo at both ends. In this approach, one end is modified with a fluorescent molecule and the other end is modified with another label that can be a quencher of fluorescence or another fluorescent probe able to accept energy from the first label. These "molecular beacons", as they are called, contain terminal sequences that are able to hybridize the ends together in solution in the absence of target. Thus, if a fluorescent molecule is at one end of the molecular beacon and a quencher is at the other end, in solution without a target DNA present the fluorescent signal would be completely quenched. In the presence of the target DNA, the molecular beacon would hybridize to the target and open up the stem-and-loop structure of the

probe, thus forcing the fluorophore and quencher too far away from each other to cause fluorescent quenching. The result is that specific target binding of the molecular beacon causes the generation of a fluorescent signal. The more fluorescence that occurs the more target is present in solution. For a review of molecular beacon technology, see Bratu (2006).

The following sections describe two methods of coupling fluorescent labels to functionalized DNA probes. Other fluorophores or quenching molecules may be attached using similar procedures with careful reference to the properties and reactivities of such labels as discussed in Chapter 9.

Conjugation of Amine-Reactive Fluorescent Probes to Diamine-Modified DNA

DNA modified with a diamine compound to contain terminal primary amines may be coupled with amine-reactive fluorescent labels. The most common fluorophores used for oligonucleotide labeling are the cyanine dyes and derivatives of fluorescein and rhodamine (Chapter 9). However, any of the amine-reactive labels discussed throughout Chapter 9 are valid candidates for DNA applications.

Some fluorescent probes are water-insoluble and must be dissolved in an organic solvent prior to addition to an aqueous reaction medium containing the DNA to be labeled. Even water-soluble fluorescent probes may be dissolved first in an organic solvent to permit easy addition to an aqueous reaction medium without hydrolysis of the reactive group. Suitable solvents are identified for each fluorophore, but mainly DMF or DMSO are used to prepare a stock solution. Some protocols utilize acetone when labeling DNA. However, avoid the used of DMSO for sulfonyl chloride compounds as this group reacts with the solvent. For oligonucleotide labeling, the amount of solvent added to the reaction mixture should not exceed more than 20 percent (although at least one protocol calls for a 50 percent acetone addition—Nicolas *et al*., 1992).

The following protocol is a generalized method for labeling amine-modified oligonucleotides with a fluorescent probe, such as FITC. It is based on the method of Morrison (1992).

Protocol

1. Prepare 10 nmol of a diamine-modified DNA probe using the chemical methods discussed in Section 2.1 or through enzymatic derivatization using an amine-containing nucleoside triphosphate (Section 1). Dissolve or buffer exchange the oligo into 1.0 ml of a suitable coupling buffer for the type of amine-reactive fluorophore utilized (see recommended reaction conditions for the particular fluorescent label in Chapter 9). For FITC, the appropriate buffer condition for the oligo is 0.1 M sodium carbonate, pH 9.0.
2. Dissolve the fluorophore in DMF or another suitable solvent at a concentration of 0.01 M. For FITC, this translates into a concentration of 3.89 mg/ml.
3. Add 50 µl of the FITC solution to the oligo solution and mix. For the use of NHS ester or sulfonyl chloride fluorescent probes, add up to 150 µl of the fluorophore solution to the DNA.
4. React overnight at room temperature.
5. Remove excess fluorophore from the labeled oligo using gel filtration on a desalting resin, dialysis, or a centrifugal concentrator.

Conjugation of Sulfhydryl-Reactive Fluorescent Probes to Sulfhydryl-Modified DNA

Fluorescent probes containing sulfhydryl-reactive groups can be coupled to DNA molecules containing thiol modification sites. The chemical derivatization methods outlined in Section 2.2 (this chapter) may be used to thiolate the oligo for subsequent modification with a fluorophore. Appropriate fluorescent compounds and their reaction conditions may be found in Chapter 9. The protocol discussed in the previous section can be used as a general guide for labeling DNA molecules.

28

Bioconjugation in the Study of Protein Interactions

The study of protein interactions has become a vital research effort as a result of the sequencing of the human genome and the genomes of other organisms. The next great challenge beyond merely having knowledge of gene sequences is to understand the complex interplay of the resultant protein molecules within cells as they bind, interact, and affect cellular processes. From the many research papers that have appeared on this subject, it is becoming clear that each protein molecule interacts with other proteins and molecules not in isolated obscurity, but in a highly complex web of interactions, which can have far reaching effects on overall biological function.

Protein interactions mediate virtually every cellular process. They are involved with transcription, translation, transport, cell cycle control, the determination of cell type and function, protein folding, post-translational modifications, signal transduction, metabolism and energy production, cell structure and motility, the formation of biological machines, cell and organism defense, and apoptosis. Genetic mutations or damaging modifications resulting in abnormal protein interactions often are the root cause of many diseases, especially tumorigenesis.

The general types of protein–protein interactions that occur in cells include receptor–ligand, enzyme–substrate, multimeric complex formations, structural scaffolds, and chaperones. However, proteins interact with more targets than just other proteins. Protein interactions can include protein–protein or protein–peptide, protein–DNA/RNA or protein–nucleic acid, protein–glycan or protein–carbohydrate, protein–lipid or protein–membrane, and protein–small molecule or protein–ligand. It is likely that every molecule within a cell has some kind of specific interaction with a protein.

The consequences of protein interactions in effecting cellular biochemistry include the synthesis, destruction, or recycling of biomolecules; the supply of energy for cellular processes; the generation of chemical signals; activation or inhibition of proteins and enzymes; changes in protein conformation and structure; the creation of new binding sites or active centers in proteins; the motility of cells, tissues, and organisms; transport of molecules within cells or into/out of cells; and the formation of cellular structures and compartments.

Protein interactions can be described in relative ways related to the strength of the binding that takes place between the two molecules and the time that the interaction lasts. Any interacting molecules can be characterized as having either (1) high affinity (strong) or low affinity (weak) binding and (2) stable (long lasting) or transient (short-lived) binding. Thus, a given

protein interaction may be described in one of four possible ways: strong and stable, strong and transient, weak and stable, or weak and transient. Interactions of the weak and stable type may at first seem like an oxymoron, but often multiple weak interactions can take place simultaneously with a target and the combined "avidity" makes the resultant complex stable.

Of the large number of protein interactions that take place in cells, perhaps the vast majority may be described as transient. Most proteins that modify other molecules do so very rapidly and so interact only briefly with their substrates or binding partners (i.e., enzymes). In addition, since proteins within cells are highly compartmentalized, the affinity of most interactions doesn't have to be very great, because each potential binding partner is within short diffusion distances and the relative concentration of molecules within these small volumes is high.

Of course, the designations of strong, weak, stable, or transient are all subjective terms. They mainly result from the outcome of affinity capture experiments of binding partners on insoluble supports or the analytical determination of the kinetic parameters of specific binding interactions. In general, if the affinity constant of a protein interaction is strong enough to allow a binding partner to be captured and purified using an immobilized affinity ligand, then the interaction can be described as being reasonably strong. This usually correlates to an affinity constant of $>10^6$/M. Conversely, interactions having affinity constants of $\leqslant 10^6$/M are often too weak to survive the washing steps needed to isolate the interacting protein on a solid phase affinity support. Quantitative measurement of the affinity constant between interacting proteins and the half-life of the interaction may be done using surface plasmon resonance (SPR) techniques (Homola *et al*., 1995; Homola *et al*., 1999).

The vast network of protein–protein interactions that have been deciphered in recent years has grown to include literally thousands of proteins in a sometimes-chaotic dance of complexity. Using the two-hybrid method for instance, which involves the use of split transcription factor fusion proteins that allow detection of interacting proteins by activation of reporter gene expression, many putative protein–protein interaction partners have been identified in yeast (Fields and Song, 1989; Chien *et al*., 1991; Criekinge and Beyaert, 1999). A map of these interactions looks a lot like a picture of the myriad nodes of addresses on a vast network of computers, such as the interconnections that make up the Internet (Jeong *et al*., 2001). Although most of the proteins in an organism have only a few partners that interact with them, major hubs in protein "interactomes" can have up to 10–20 links with other proteins, indicating that these key proteins are critical players in cell vitality Figure 28.1.

Through the growing knowledge of protein–protein interactions and their corresponding gene sequences major interaction domains on protein surfaces are being identified. These relatively conserved amino acid sequences create structural motifs that are designed to bind with certain targeted sequences in other proteins or molecules (Pawson and Nash, 2003; Ingham *et al*., 2005). Using this information, many common binding regions on proteins can be identified just through their gene sequences.

However, it is much more difficult to characterize the interactions of proteins with no known interaction domains. The traditional "lock and key" approach to conceptualizing binding pairs is far too simplistic to allow easy visual identification of interacting surfaces on the complex three-dimensional space making up the topography of protein molecules. Even in those instances where protein interacting partners have been crystallized together and their three-dimensional structures determined, it's obvious from the molecular models that it would be difficult or impossible to identify visually the site of interaction without having such structural data in place beforehand. For this reason, experimental schemes are needed that are more

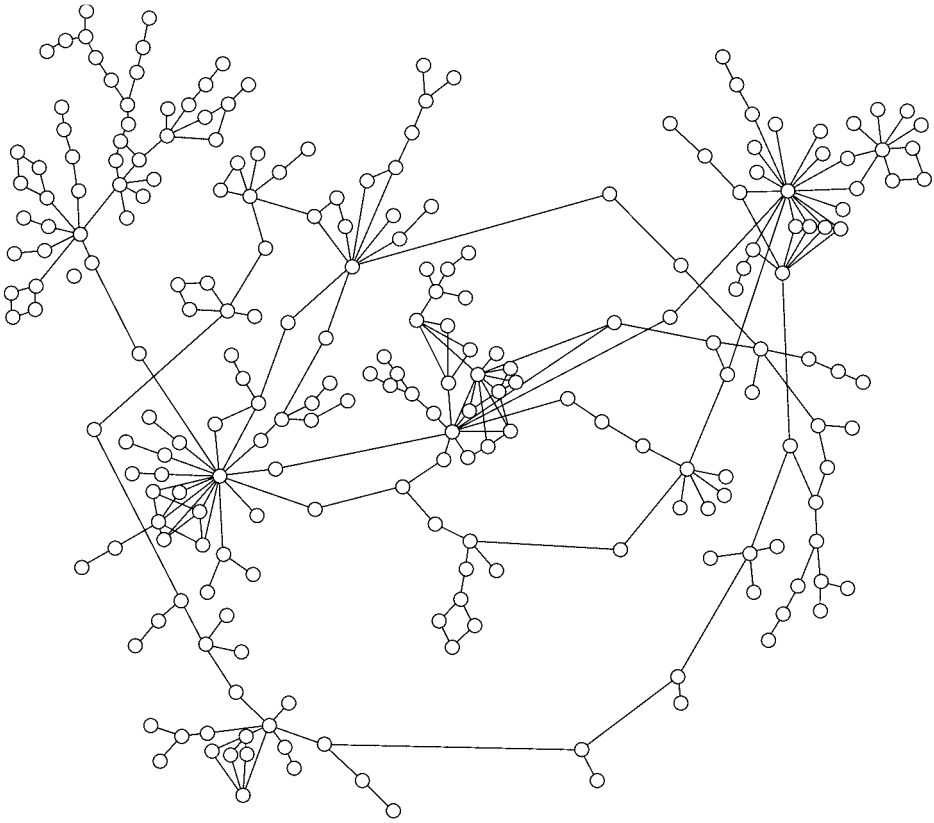


Figure 28.1 A small segment of the yeast interactome. The spheres represent proteins and the interconnecting lines are identified protein interactions. Many proteins are seen to interact with one or two other proteins, but some can have over a dozen other interacting partners.

elaborate than just knowledge of genetic sequences or interaction domains to characterize the majority of specific interactions proteins undergo. Even when two-hybrid studies indicate the probability of a protein interaction occurring, it still doesn't provide information on the nature of the interaction, the binding sites, or its function.

The techniques developed to study protein interactions can be divided into a number of major categories (Table 31.1), including bioconjugation, protein interaction mapping, affinity capture, two-hybrid techniques, protein probing, and instrumental analysis (i.e., NMR, crystallography, mass spectrometry, and surface plasmon resonance). Many of these methods are dependent on the use of an initial bioconjugation step to discern key information on protein interaction partners.

Many of the methods developed to study protein interactions use the bait/prey model to detect interacting partners (Phizicky and Fields, 1995; Archakov *et al*., 2003 Piehler, 2005). The bait protein is a purified protein (often recombinant) that is used to lure and capture a putative interacting protein or biomolecule. The bait protein may be immobilized to a solid phase for affinity separations or be used in solution. It also may be fusion tagged (i.e., GST or 6× His) or labeled with a detectable molecule, such as a fluorescent probe. It often is the case

that there exists an antibody specific for the bait protein to use for detection or in recovery of the interacting complexes.

The prey is a protein, protein complex, or other biomolecule that interacts with the bait protein. It can be captured by its specific affinity interaction with the bait. Since many protein interactions may involve low affinity binding events or are transient, the use of chemical crosslinking techniques can greatly facilitate analysis of an interacting prey protein. In fact, the use of bioconjugation to "fix" interacting proteins is a powerful route to capturing weak affinity or transiently interacting molecules, as the crosslinked complexes can be isolated and analyzed without loss of some components.

The following sections describe the use of bioconjugation reagents for the study of protein interactions. These reagents and techniques can be used to crosslink and capture interacting proteins, to investigate the binding sites involved with interactions, and to identify which peptide regions or amino acids participate in the binding event. In addition to the bioconjugation reagents described in this section, the reader also is referred to Chapter 16, entitled Mass Tags and Isotope Tags, wherein some of those reagents also can be used to investigate interacting proteins.

1. Homobifunctional Crosslinking Agents

One of the first applications of bioconjugate techniques was the use of simple homobifunctional compounds (see Chapter 4) to capture interacting proteins in biological samples. As the name of this type of reagent implies, these compounds have reactive groups on each end of a spacer arm that are identical and react with the same type of functionality on different proteins. For instance, early development of crosslinking compounds resulted in the creation of homobifunctional amine-reactive reagents using either imidoesters or NHS esters as the reactive groups. Both of these reagent types could be reacted with a complex protein sample mixture to result in efficient conjugation of proteins through their available amine groups. The reactive groups on these compounds survive long enough in a physiological pH environment to effectively capture and covalently link proteins in proximity to one another. In this way, any proteins undergoing specific biological interactions can be "fixed" by chemical conjugation and thus link together permanently interacting complexes for analysis.

However, as one might expect, this strategy is more or less a crude "shotgun" approach, wherein every protein in the sample has the potential to be crosslinked with other proteins regardless of whether they are undergoing specific interactions or not. The use of homobifunctional reagents to study protein interactions therefore may result in high noise levels or many false positives, because of their non-selective crosslinking characteristics. The end result often makes it difficult to identify conjugated proteins that are specifically interacting from those proteins crosslinked just due to random collisions.

However, even acknowledging their disadvantages, homobifunctional crosslinkers have been used successfully to investigate many protein–protein interactions, within cells and within lysates or protein solutions. The key to capturing true interacting proteins while limiting the degree of nonspecific conjugation is to optimize the amount of crosslinker at the lowest possible concentration. Using too high a concentration will extensively conjugate all proteins, even those that are not specifically interacting at the moment. Using too low a concentration will not conjugate effectively even interacting proteins. Therefore, optimization typically is needed to determine the best concentration of homobifunctional crosslinker for a particular application.

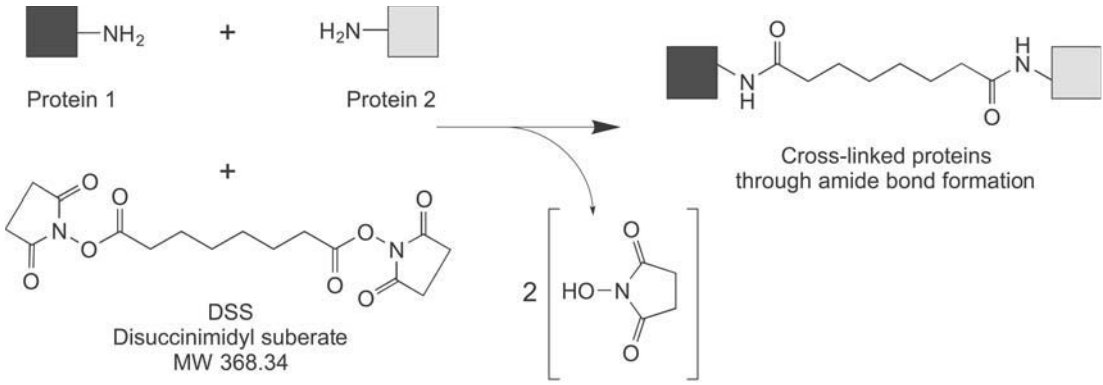


Figure 28.2 DSS can capture protein interacting partners through amide bond crosslinks.

1.1. DSS and BS^3

Two crosslinking agents that have been used extensively to study protein interactions are disuccinimidyl suberate (DSS) and bis-sulfosuccinimidyl suberate (BS^3) (see Chapter 4, Section 1.2). Both reagents contain an 8-carbon spacer arm built from suberate core and have reactive esters at each end that couple with amines to form amide bonds. They differ, however, in the fact that DSS contains NHS esters and BS^3 contains negatively charged sulfo-NHS esters. BS^3 therefore is water-soluble and will not penetrate cell membranes, whereas DSS is hydrophobic, water-insoluble, and is membrane permeable. For this reason, BS^3 can be used to study cell-surface–protein interactions (Friedrichson and Kurzchalia, 1998; Simons *et al.*, 1999), while DSS can be used to study intracellular protein interactions (Ishmael *et al.*, 2005). The reaction of DSS to capture and crosslink interacting proteins is shown in Figure 28.2.

The following protocol represents a generalized strategy for crosslinking interacting proteins using either DSS or BS^3. The buffer conditions and reagent amounts are gleamed from published procedures, but the exact quantities should be optimized for each protein interaction studied.

Protocol

1. Suspend cells at ~25×10^6 cells/ml in PBS (pH 8.0).
2. Wash cells 3 times with ice-cold PBS (pH 8.0) to remove amine-containing culture media and extracellular proteins from the cells.
3. For cell-surface interaction studies, add ligands to the cells and incubate for 1 hour at 4°C.
4. Dissolve DSS or BS^3 in dry DMSO at a concentration of 25 mM. Note: BS^3 may be added directly to PBS buffer or dissolved as a stock solution in DMSO.
5. Add an aliquot of the DSS or BS^3 solution to the reaction medium to obtain a final concentration of 0.5–5 mM. *Note*: Simons *et al.* (1999) successfully used a concentration of 0.5 mM BS^3 with Madin-Darby canine kidney (MDCK) cells permanently expressing a GPI-anchored form of growth hormone decay accelerating factor (GH-DAF) to crosslink the protein interaction complexes on the cell surfaces.

6. Incubate the reaction mixture for 30 minutes at room temperature. To reduce active internalization of BS^3 into cells, this incubation may be performed at 4°C.
7. Quench the reaction by adding an aliquot of 1 M Tris, pH 7.5, to give a final concentration of 10–20 mM.
8. Incubate the quenching reaction for 15 minutes at room temperature.
9. Lyse cells and analyze the protein interactions by electrophoresis, Western blotting, and mass spec.

1.2. Heavy Atom, Deuterated Crosslinking Agents

Another approach to the study of protein interactions using homobifunctional crosslinkers uses the incorporation of isotopes, like deuterium, into the reagent structure to produce a heavy atom analog suitable for mass spec analysis. In this technique, the normal or light H (hydrogen) version of a crosslinker is used in an equal molar ratio to a heavy D (deuterium) version to capture interacting proteins through covalent conjugation. The heavy and light analogs are reacted with a sample at the same time, so that each form of the reagent will have an equal chance of reacting with an interaction complex. Subsequent proteolytic digestion of the sample (i.e., with trypsin) creates peptide fragments, some of which will contain covalent crosslinks from the heavy or light crosslinkers. In addition, these crosslinked peptides will have an equal chance of having either a light atom linker or a heavy atom linker holding them together.

Mass spec analysis of the peptide fragments formed by this process yields pairs of MS peaks differing only by the mass change caused by the substitution of deuterium atoms for hydrogen atoms in half of the crosslinks. Thus, searching for MS peaks in the data that differ by the number of deuterium substitutions immediately will identify peptides from the interacting proteins that have been captured by the crosslinking process.

Using this approach, homobifunctional crosslinking agents containing sulfo-NHS esters have been developed based on the core structures of glutaric and suberic acids. The suberate-based crosslinker also is known as BS^3 and has been described previously (Section 1.1, this chapter). These two standard amine-reactive reagents then are modified at two carbons of their respective cross-bridge structures to contain two pairs of deuterium atoms, increasing their molecular mass by exactly 4 from the normal hydrogen atom analogs.

These heavy atom reagent pairs are termed $BS^2G\text{-}d_4$ (bis[sulfosuccinimidyl] 2,2,4,4 glutarate-d_4) and $BS^3\text{-}d_4$ (bis[sulfosuccinimidyl] 2,2,7,7 suberate-d_4), and their light atom analogs are called $BS^2G\text{-}d_0$ (bis[sulfosuccinimidyl] glutarate-d_0) and $BS^3\text{-}d_0$ (bis[sulfosuccinimidyl] suberate-d_0) (Thermo Fisher) (Figure 28.3). The sulfo-NHS esters of these four compounds all react equally well with amine groups on proteins to form amide bonds. Therefore, there are virtually no differences in properties or reactivities between the heavy and light versions of these reagents.

The conjugation of interacting proteins with heavy/light crosslinkers potentially can result in a number of derivatives having unique atomic mass units (amu) observed by mass spec. For instance, any of these crosslinkers might react with amines on interacting proteins at both ends to form amide bonds. Alternatively, one end of these homobifunctional compounds may react with a protein to form an amide bond while the other end hydrolyzes, resulting in loss of the sulfo-NHS group to form a carboxylate. Thus, the heavy/light crosslinking pairs may have two

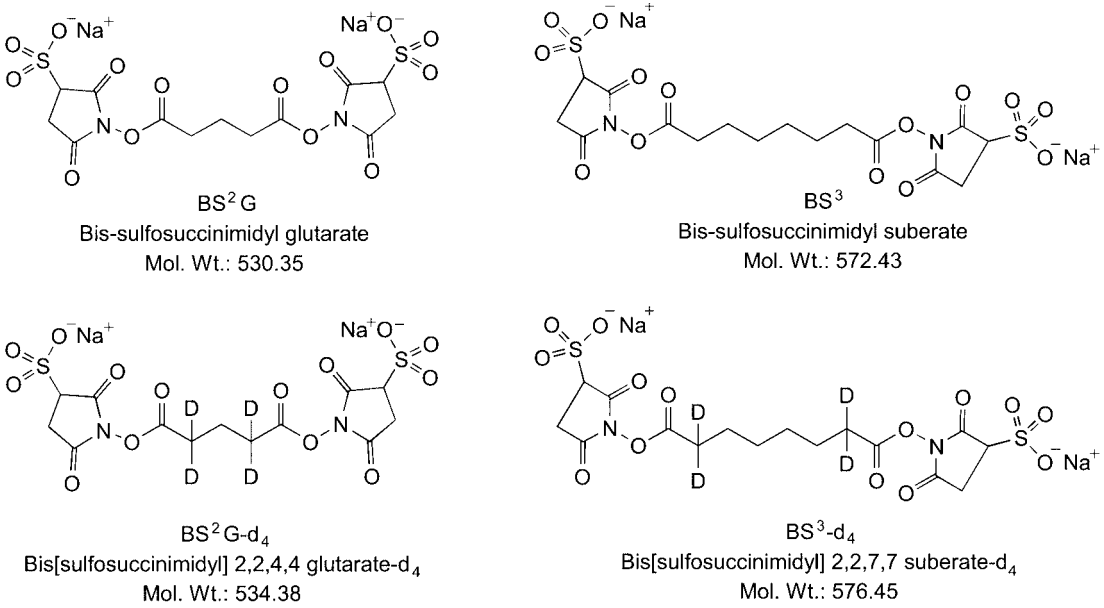


Figure 28.3 The homobifunctional crosslinkers BS^2G and BS^3 can be used to capture protein interactions through amide bond formation. The deuterium-labeled analogs of these reagents can be used to differentiate samples by mass spec.

potential amu results when peptides are analyzed by mass spec. Table 31.2 shows the potential amu contributions for each of the possible products formed by the reactions of the heavy/light crosslinkers with proteins.

This technique has been described as a general method of studying protein–protein interactions as well as a method for investigating the three-dimensional structure of individual proteins (Muller *et al.*, 2001; Back *et al.*, 2003; Dihazi and Sinz, 2003; Sinz, 2003; Sinz, 2006). It also has been used for the study of the interactions of cytochrome C and ribonuclease A (Pearson *et al.*, 2002), to investigate the interaction of calmodulin with a specific peptide binder (Kalkhof *et al.*, 2005a; Schmidt *et al.*, 2005), and for probing laminin self-interaction (Kalkhof *et al.*, 2005b).

In practice, sample concentrations typically are kept in the micromolar range to limit the widespread conjugation of molecules not specifically interacting or to limit intermolecular conjugation when studying the structure of a single protein. Therefore, most protein concentrations will be in the microgram/ml range prior to the addition of crosslinkers. Even in cases wherein a single purified protein is crosslinked to study its three-dimensional structure, limiting the amount of crosslinker in the reaction mixture will help to limit polymerization of the protein. However, successful results have been obtained by this method using up to a 200-fold excess of crosslinker over the concentration of protein in solution.

The following protocol is based on the published methods as well as the specific instructions provided with the heavy atom crosslinkers from Thermo Fisher Scientific. For new applications, the amount of crosslinkers added to the sample will have to be optimized to obtain useful information about the interactions.

Protocol

1. Dissolve the protein(s) to be studied in 20 mM HEPES buffer, pH 7.5, at a concentration of 5–10 μM.
2. Dissolve together in one solution the desired heavy and light crosslinker analogs in dry DMSO at an equal concentration of up to 100 mM. Use either the pair $BS^2G\text{-}d_4/BS^2G\text{-}d_0$ or $BS^3G\text{-}d_4/BS^3G\text{-}d_0$, but do not mix the different sized crosslinkers together. The heavy and light analogs of the same type should always be dissolved at equivalent concentrations in DMSO to prepare the stock solution.
3. Add a quantity of the crosslinker solution to the protein solution to obtain at least a 10-fold molar excess of the crosslinkers over the concentration of the protein. Studies should be done at several levels of crosslinker addition to determine the optimal conjugation conditions (i.e., 10-, 50-, 100-, and 200-fold excess).
4. Quench the reaction by the addition of NH_4HCO_3 to a final concentration of 20 mM. The optimal time course for the reaction should be determined by removing portions of the solution at different points, starting at about 5–10 minutes and extending out to 2 hours in length.
5. Analyze the quenched reaction by SDS electrophoresis, Western blotting, and mass spec analysis.

1.3. Formaldehyde

By Far the simplest bifunctional crosslinking agent is formaldehyde. Although structurally a mono-functional aldehyde compound, formaldehyde reacts with proteins via a two-step reaction

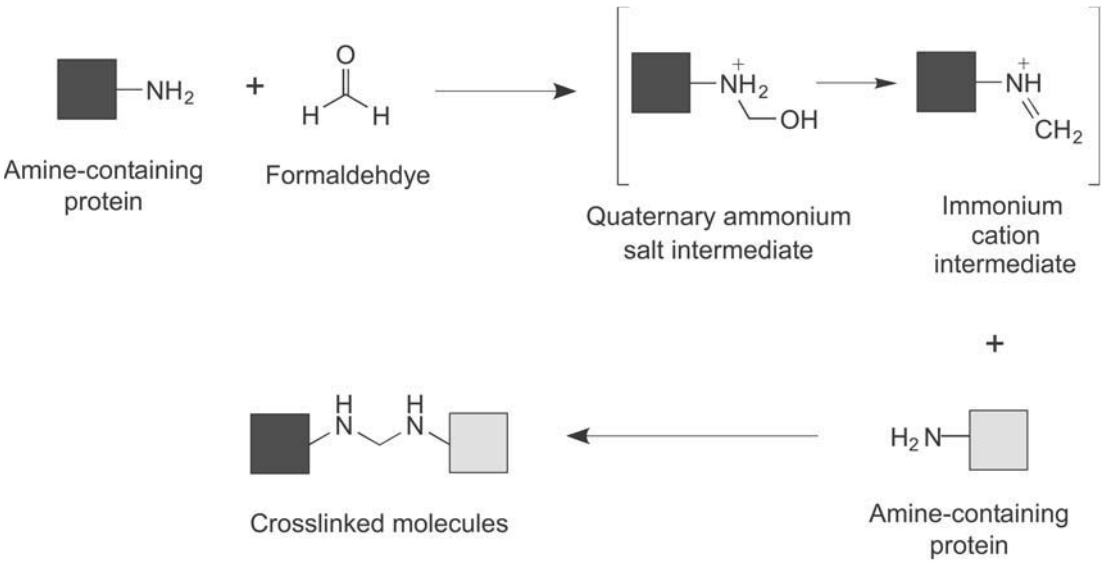


Figure 28.4 Formaldehyde can be used to capture protein interactions if it is used at low concentrations. The reaction proceeds through modification of a protein to create an intermediate immonium cation, which then goes on to react with a neighboring protein to form the crosslinked product via secondary amine bonds.

to yield a methylene bridge crosslink between two amines on proteins and other molecules, thus it behaves as though it is bifunctional in nature (Figure 28.4). The reaction of formaldehyde with proteins is rapid and efficient, and it has long been used to fix cells and tissue samples for preservation, staining, and probing. Formaldehyde quickly penetrates cells and at the right concentration, results in locking biomolecules in place, preventing diffusion, morphological changes, or loss of proteins through solubilization.

Formaldehyde also can be used at limiting concentrations to crosslink interacting proteins, while avoiding the extensive global crosslinking typically obtained when fixing cells. At relatively low concentrations, formaldehyde will link together only those proteins and other amine-containing molecules within proximity to one another and presumably, therefore, undergoing specific biological interactions (Prossnitz *et al*., 1988; Skare *et al*., 1993; Derouiche *et al*., 1995; Orlando *et al*., 1997; Orlando, 2000; Hall and Struhl, 2002; Vasilescu *et al*., 2004).

Guerrero *et al*. (2006) used this technique along with the quantitative mass spec strategy called SILAC (stable isotope labeling of amino acids in cell culture; Ong *et al*., 2002) to identify the yeast proteins that interact with the 26 S proteasome.

The following protocol is a generalized method that summarizes the publications on the use of formaldehyde for capturing interaction proteins. The ranges indicated for concentrations of reactants and time of the reaction need to be optimized for each protein interaction studied.

Protocol

1. Grow cells and wash into ice-cold 10 mM PBS buffer, pH 6.8.
2. Add formaldehyde to a final concentration of 0.125–1 percent (w/w) (optimize to find the best concentration level for the particular protein being studied).
3. React at room temperature to 37°C for 5–60 minutes (optimize to find the best reaction time for the protein being studied).
4. Wash cells and solubilize pellet in SDS-PAGE sample buffer.
5. Heat at 37°C for 10 minutes to fully solubilize and maintain crosslinked proteins, and then enrich specific complexes by immunoprecipitation using an immobilized antibody specific for the bait protein that was used. Alternatively, heat at 96°C for 20 minutes to solubilize and break all crosslinks (this may be used as a control).
6. Analyze interacting proteins by electrophoresis, Western blotting, and mass spectrometry.

1.4. Protein Interaction Reporters

Standard homobifunctional crosslinkers, such as those described in the previous sections, can capture protein interactions effectively through covalent linkages, but they create severe challenges for analyzing exactly what proteins have been conjugated. This is not just the result of the large number of protein interacting partners that get crosslinked; it is also a result of the wide variety of products that can form from the process, including side reactions, nonspecific conjugations, and crosslinkers that only reacted with one protein. Attempting to deconvolute the identity of true protein interacting complexes created by large-scale crosslinking of complex samples is the major problem of all conjugation techniques used to study protein interactions.

A new type of crosslinking strategy may overcome this problem, as it takes advantage of definitive mass spec identification after the chemical crosslinking of interacting proteins. The

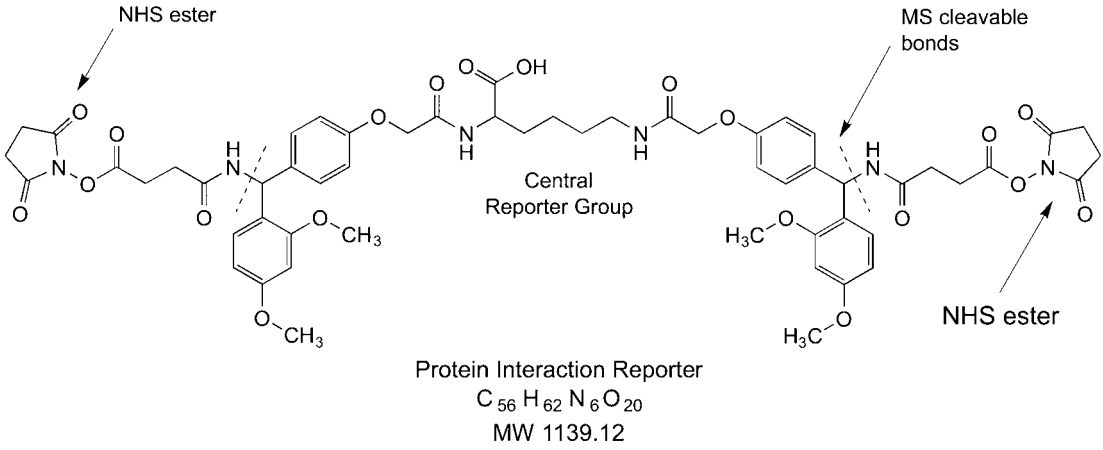


Figure 28.5 This PIR compound contains NHS esters at both ends to capture interacting proteins through amide bond formation. It also contains MS cleavable bonds that release a central reporter group, which can be used to identify crosslinked peptides by mass spec.

protein interaction reporter (PIR) reagent as described by Tang *et al.* (2005) is based on a bifunctional crosslinker concept, but having the additional feature of containing two mass spec cleavable bonds within a specially designed spacer arm. The cleavable parts of the molecule consist of two RINK groups, which are amide-releasing, acid-cleavable components based on the solid phase peptide synthesis resin first described by Rink (1987), and containing the 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy linker on each side of a central mass reporter tag (Figure 28.5). On both ends of the PIR compound, NHS esters provide amine reactivity to capture any interacting proteins in a complex sample, such as within a cell or lysate. In use, the PIR crosslinker first is reacted with a sample to form conjugates and then the proteins are subjected to proteolysis to create peptides. Some of the peptides that are formed will be conjugated together through the PIR crosslinks. This complex mixture of peptides then is subjected to mass spec analysis using a mass spectrometer capable of MS^2 or MS^3 separations.

Second-generation PIR reagents have been designed to include an affinity handle branching off from the central reporter group's free carboxylate. In one such design, a biotin group is built at the end of a hydrophilic PEG spacer (Figure 28.6). The PEG arm provides increased water-solubility to the overall reagent, which is otherwise rather hydrophobic. The biotin group can be used to detect or capture crosslinked interacting proteins out of complex solutions. For instance, immobilized streptavidin can be used to pull down any proteins modified with the biotin–PIR reagent. Alternatively, a complex solution may be separated by electrophoresis and the modified proteins identified after Western blotting through the use of a streptavidin conjugate detection complex.

The use of a crosslinker containing MS labile bonds along with a mass reporter group can significantly reduce the complexity of finding and identifying coupled peptides in a huge amount of mass spec data. In the first dimension of a MS separation, the overall mass of the PIR-crosslinked peptides can be accurately determined as a single peak in the MS spectrum. The resultant mass correlates to the sum of the two crosslinked peptides plus the intervening PIR spacer which links them together.

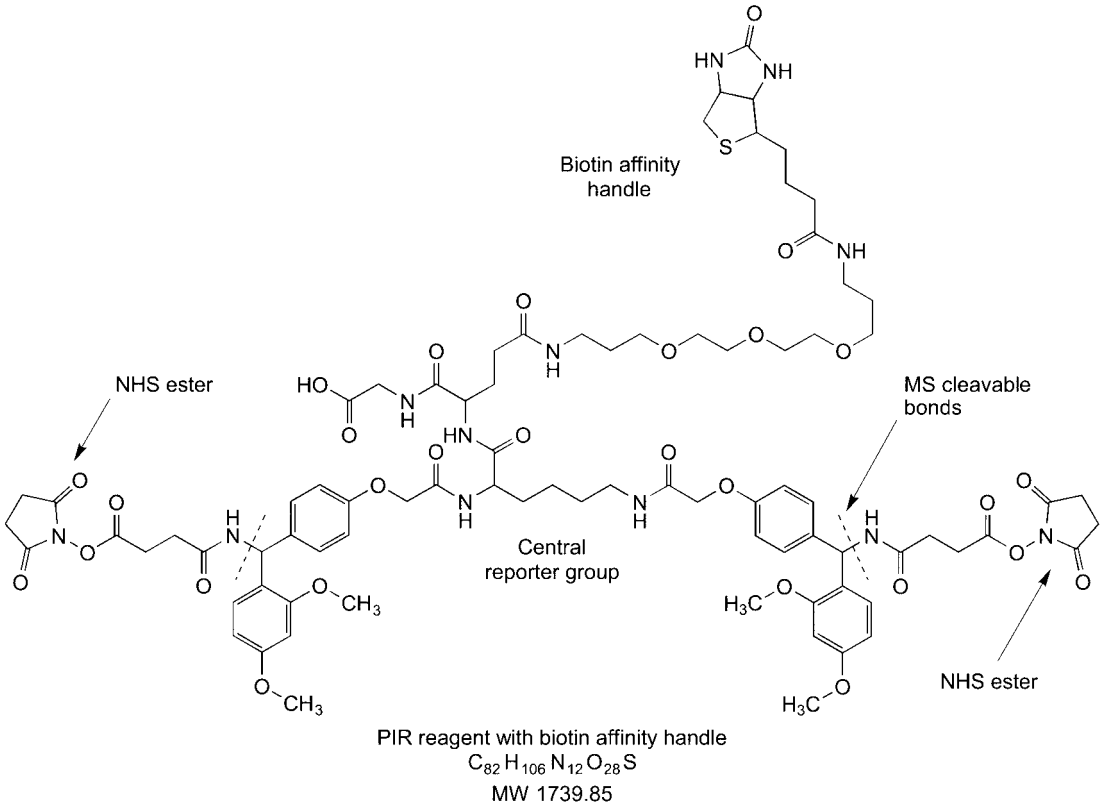


Figure 28.6 A trifunctional PIR compound that contains two NHS esters to capture interacting proteins through amide bond formation and a PEG–biotin arm to permit isolation of crosslinked proteins on (strept)avidin supports.

With bifunctional NHS ester reagents, one of three modification products can occur with proteins: (1) a dead-end linkage wherein one end of the crosslinker has attached to an amine group within a protein and the other end has hydrolyzed and not formed an attachment, (2) an intra-protein crosslink wherein the PIR reagent has been coupled at both ends to amines within the same protein, or (3) an inter-protein crosslink wherein both ends of the PIR reagent have been coupled with amines on two different protein molecules (Figure 28.7).

The second stage of an MS^2 analysis can be done to bombard with more energy the mass products of the first stage separation in order to fragment the proteolytically digested complexes. At this point, the mass reporter group within the PIR crosslink is released due to breakdown of the two labile RINK bonds within the reagent structure (see Figure 28.5). Mass spec cleavage of these groups and release of the reporter results in one of two potential mass signatures depending on if both ends of the PIR reagent have reacted (which gives a reporter mass = m/z 711) or if only one end has reacted (a dead-end; reporter mass = m/z 828). The resulting mass spectrum then shows either two or three peaks, depending on the type of modification formed from the initial PIR reaction. This process also releases the crosslinked peptides without disrupting the

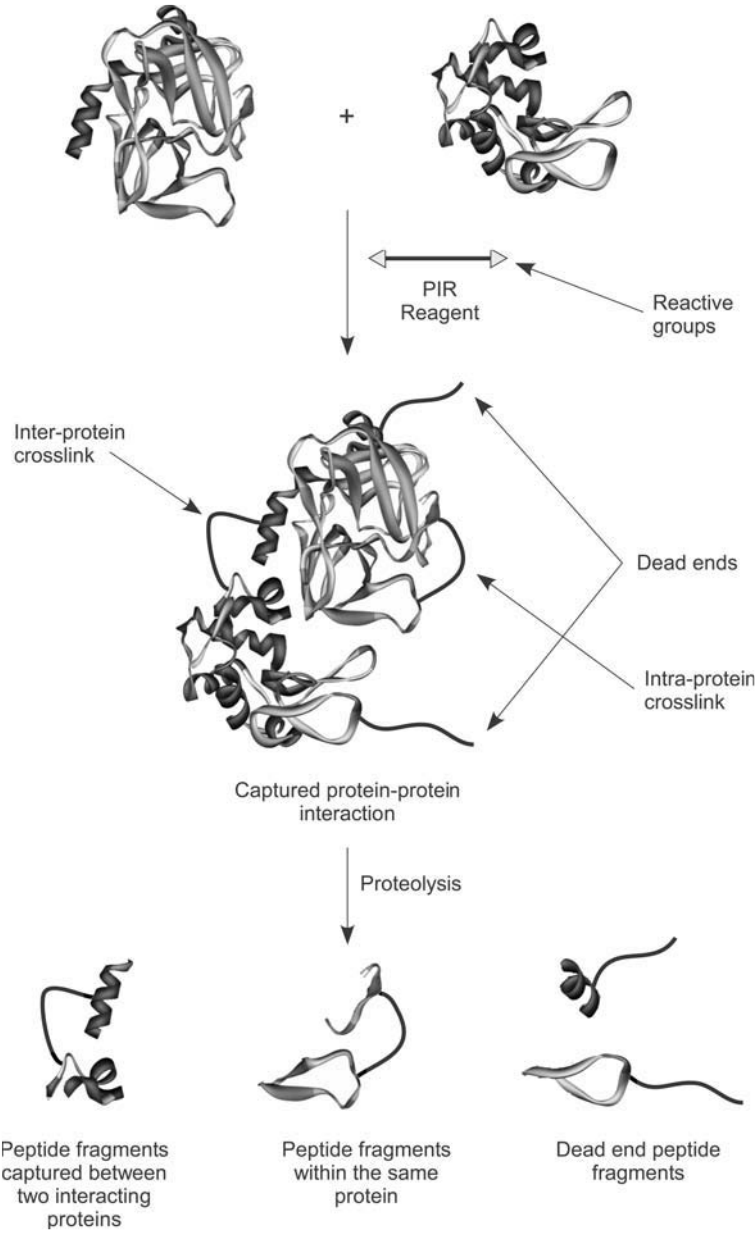


Figure 28.7 Reaction of a PIR compound with a protein sample can result in several products, all of which can be identified by mass spec analysis. A true inter-protein crosslink can occur that links two interacting proteins together, which is the desired product. However, the crosslinking process also may result in intra-protein crosslinks or dead ends wherein only one end of the PIR reagent has coupled to a protein.

peptide backbone and thus leaves a small part of the PIR compound attached to each peptide fragment, minus the reporter group.

The specific fragmentation pattern observed at this stage distinguishes the kind of crosslink or modification that was initially formed. In this regard, a single modified peptide peak plus a reporter peak of mass m/z 828 indicates a dead-end modification with no value in determining protein interactions. Alternatively, a single peptide fragment peak plus a reporter group peak of mass m/z 711 indicates an intra-molecular crosslink made between regions of the same protein, which also is not of interest. However, a fragmentation pattern containing two labeled peptide peaks plus a reporter peak of mass m/z 711 indicates a successful conjugation between two protein molecules, which may be indicative of a true protein–protein interaction. Note that alternative designs of a PIR-type reagent containing other reporter structures, including those with a biotin handle and a PEG spacer, will result in different reporter fragmentation mass values than those stated here.

The use of PIR compounds to study protein interactions is a significant advance over the use of standard homobifunctional crosslinkers. The unique design of the PIR reagent facilitates deconvolution of putative protein interaction complexes through a simplified mass spec analysis. The software can ignore all irrelevant peak data and just focus analysis on the two labeled peptide peaks, which accompany the reporter signal of appropriate mass. This greatly simplifies the bioinformatics of data analysis and provides definitive conformation of protein–protein crosslinks.

Finally, knowledge of the peptide masses that resulted from the PIR conjugation provides information to identify the parent proteins from which they originated. Peptide mass and sequence databases now are sufficiently developed to provide rapid confirmation of protein–protein interaction partners.

The following protocol is designed for treating cells with the PIR reagent to study protein interactions *in vivo*. It is based on the method of Tang *et al.* (2005). The use of the PIR compound to treat intact cells results in the crosslinking of proteins both on the cell surface and within the cell, which indicates that the reagent is able to cross the cell membrane.

Protocol

1. Dissolve the PIR compound in dry DMSO to make a 100 mM stock solution.
2. Grow cells in media to a density of about OD_{600nm} = 1.2 and harvest in mid-log phase. Centrifuge cells at 3,200 rpm to pellet them and wash 3 times with ice-cold PBS (150 mM sodium phosphate, 100 mM NaCl, pH 7.5).
3. Suspend the cells in 1 ml of PBS and add an aliquot of the dissolved PIR compound to bring the final concentration to 1 mM.
4. React at room temperature with gentle shaking for 5 minutes.
5. Quench the reaction by the addition of 50 µl of 1 M Tris, pH 7.5.
6. Wash the cells 5 times by centrifugation with cold PBS to remove excess PIR reagent and any secreted proteins.
7. Lyse the cells using a detergent lysis buffer suitable for the cell type being treated. Centrifuge the lysate at 15,000 rpm for 40 minutes at 4°C to remove insoluble material. Collect the supernatant and discard the pellet. At this point, the soluble protein fraction may be analyzed by electrophoresis, if desired.
8. Remove unreacted PIR reagent and reaction by-products by gel filtration or dialysis.
9. Precipitate the protein with TCA to further remove any remaining salts and detergent. Centrifuge to pellet the precipitated protein, wash the pellet with TCA, and centrifuge again. Redissolve the washed pellet in 100 µl of 100 mM NH_4HCO_3, pH 7.8, containing 8 M urea.

10. Reduce protein disulfides by adding dithiothreitol (DTT) to a final concentration of 5 mM and incubate for 30 minutes at 60°C. Add iodoacetamide to a final concentration of 25 mM to alkylate the thiols. React for 1 hour in the dark.
11. Dilute the solution 4-fold with 100 mM 25 mM NH_4HCO_3, and then add 20 µg trypsin to digest the proteins. Incubate at 37°C for 4 hours or overnight at 30°C with shaking.
12. Purify the tryptic peptides by chromatography on a C18 column to remove salts (follow the manufacturer's directions for peptide purification). Dry the eluent and redissolve the peptides in 20 µl 0.1 percent formic acid.
13. Analyze the purified peptides using two-dimensional LC–MS/MS.

2. Use of Photoreactive Crosslinkers to Study Protein Interactions

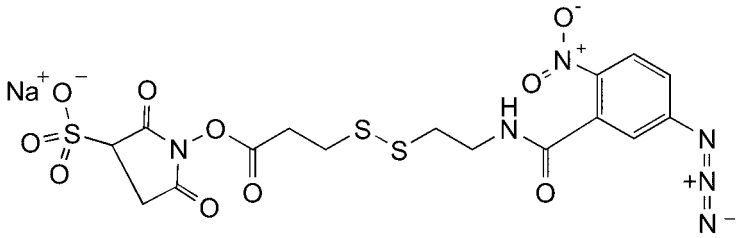
The use of crosslinking agents containing at least one photoreactive group provides reagents that can be activated at a desired point after bait proteins have been allowed to interact and bind to prey proteins. The availability of the first photoreactive homobifunctional or heterobifunctional compounds permitted protein interactions to be studied at a new level of specificity. Unlike the use of spontaneously reactive homobifunctional reagents, the incorporation of a photoreactive group helps to prevent nonselective crosslinking and uncontrolled polymerization of proteins that may or may not be specifically interacting.

In use, a bait protein first is modified with the spontaneously reactive (thermoreactive) end of a photoreactive heterobifunctional compound, while protecting the solution from light exposure. The modified bait protein then is allowed to interact with a sample containing other proteins and biomolecules, which may contain prey proteins able to interact with it. Finally, the sample mixture is exposed to UV light to activate the photoreactive end of the crosslinker, causing conjugation of this group with any nearby interacting molecules. The rapid reaction rate of the activated photoreactive intermediates assures that they don't survive long enough to cause much conjugation to proteins just due to random collisions. Interacting proteins, however, that are in proximity to the modified bait protein are more likely to be captured through reaction with the activated photoreactive group.

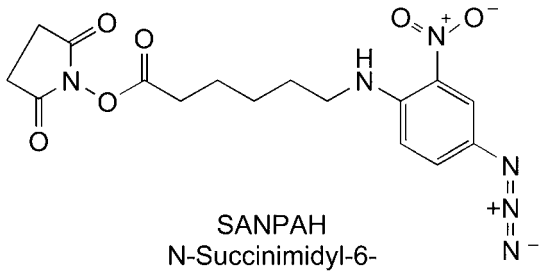
The following sections discuss the application of several photoreactive heterobifunctional crosslinkers to the study of protein interactions.

2.1. Sulfo-SAND, SANPAH, and Sulfo-SANPAH

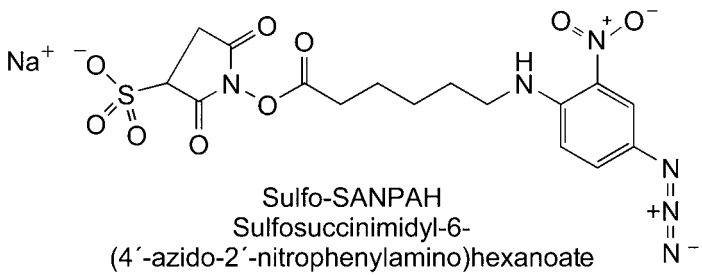
These three photoreactive crosslinkers are described in terms of their properties and reactivities in the section on Amine-Reactive and Photoreactive Crosslinkers in Chapter 5, Section 3. They represent early examples of the use of standard phenyl azide photoreactive compounds for the study of protein interactions. All of them either contain an amine-reactive NHS ester or the charged and water-soluble analog, a sulfo-NHS ester (Figure 28.8). SANPAH is uncharged and hydrophobic and thus provides membrane permeability for studying intracellular protein interactions in whole cells (Mikhailov *et al.*, 2001; Kota and Ljungdahl, 2005). Sulfo-SAND and Sulfo-SANPAH, however, possess a negatively charged sulfonate group, which prevents them from passing through cell membranes, and thus are membrane impermeable (Uckun *et al.*, 1995; Gaudet, *et al.*, 2003). The sulfonated compounds may be used with whole cells to study



Sulfo-SAND
Sulfosuccinimidyl 2-(*m*-azido- o -nitrobenzamido)
ethyl-1,3′-dithiopropionate
Mol. Wt.: 570.51



SANPAH
N-Succinimidyl-6-
(4′-azido-2′-nitrophenylamino)hexanoate
Mol. Wt.: 390.35



Sulfo-SANPAH
Sulfosuccinimidyl-6-
(4′-azido-2′-nitrophenylamino)hexanoate
Mol. Wt.: 492.40

Figure 28.8 The heterobifunctional crosslinkers sulfo-SAND, SANPAH, and sulfo-SANPAH contain an amine-reactive (sulfo)NHS ester on one end and a photoreactive phenyl azide group on the other end. Sulfo-SAND allows release of conjugates by reduction of its internal disulfide bridge.

cell-surface protein interactions, although in use, their concentration should be limited to prevent internalization by active transport into cells.

Sulfo-SAND, SANPAH, and Sulfo-SANPAH all contain a nitrated phenyl azide photoreactive group. The presence of the nitro group shifts the optimal wavelength for photoactivation

to higher wavelengths, thus avoiding the potential for biomolecule damage due to UV irradiation (photoactivation occurs at 320–350 nm). Sulfo-SAND provides one further option for studying interacting proteins. It has a cleavable disulfide containing cross-bridge that permits recovery of any prey proteins that have been captured by the photo-coupling process (McMahan and Burgess, 1994; Uchiyama *et al*., 2002). Finally, each of these photoreactive crosslinkers has phenyl azide rings that undergo ring expansion to the 7-membered ring, dehydroazepine intermediate, which reacts primarily with amines on target molecules. Thus, the proteins that are captured by these crosslinkers typically are coupled through secondary amine linkages to the photoreactive end of the reagents. A general protocol for the use of these compounds to study protein interactions is given at the end of the next section.

2.2. Sulfo-SFAD

Sulfo-SFAD is sulfosuccinimidyl-[perfluoroazidobenzamido]-ethyl- 1,3′-dithiopropionate, an amine reactive and photoreactive crosslinker with more advanced properties than the photoreactive reagents discussed above. This reagent contains a sulfo-NHS ester to provide increased water-solubility prior to conjugation, and this group will react with an amine on proteins and other biomolecules to form an amide linkage. Sulfo-SFAD also contains a perfluorophenyl azide group that has better photo-insertion capability than the original unsubstituted phenyl azide group. The reason for this enhanced yield is that after photoactivation, the nitrene intermediate can't react with the phenyl ring itself and undergo ring expansion to a dehydroazepine as happens with typical unsubstituted phenyl azides. The result is that the nitrene survives long enough to react with neighboring molecules in the immediate molecular vicinity, such as proteins that are interacting with the modified bait protein. Perfluorophenyl azides thus have higher yields of conjugation, and they can react by insertion into C—H bonds, N—H, unsaturated carbon–carbon bonds, and other structures in target molecules. By contrast, unsubstituted phenyl azides ring expand and react mainly with amines, and even then at low yield. The perfluorophenyl azide may be activated with UV light at 320 nm.

Sulfo-SFAD also contains a disulfide bond in its cross-bridge, which provides subsequent cleavability of any crosslinks formed. Thus, prey proteins can be recovered from complexes for analysis by simple reduction of the conjugate with DTT. Once reduced, the perfluorophenyl ring group of Sulfo-SFAD is transferred to the interacting prey protein. This group can be identified by ^{19}F NMR, and it also provides a traceable label for identification of peptide fragments by mass spec. Figure 28.9 illustrates the reactions of Sulfo-SFAD in capturing a bait–prey complex.

The following references provide further information on the use of Sulfo-SFAD and perfluorophenyl azide photoreactive groups: Pandurangi *et al*. (1995a, b, 1996, 1997a, b, 1998); Yan *et al*. (1994).

A general protocol that can serve as a guide for the use of heterobifunctional crosslinkers in the study of protein interactions is given below. Some optimization of concentrations may have to be done depending on the particular type and properties of proteins being studied.

Protocol

1. Dissolve the photoreactive crosslinker in DMF or DMSO at a concentration of 10–25 mM (protect from light). Water-soluble crosslinkers can be added to water or buffer, but should be used immediately.

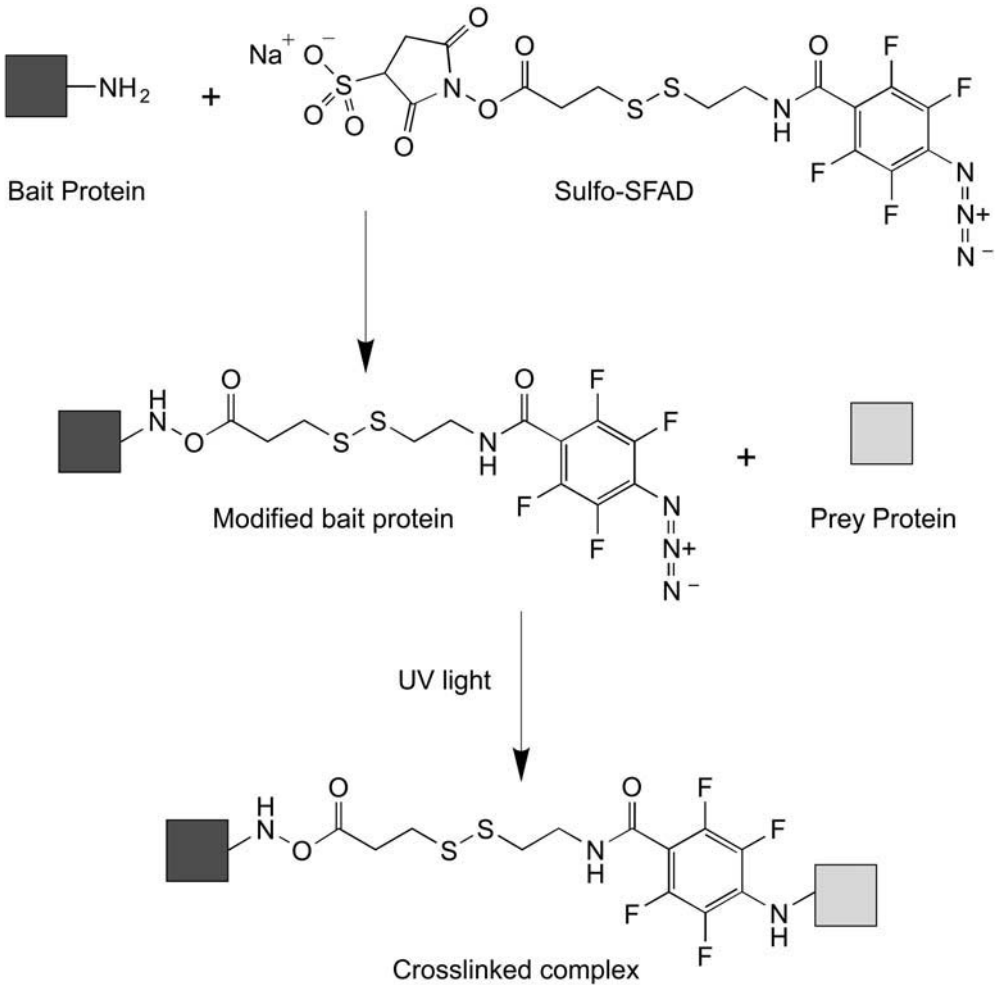


Figure 28.9 Sulfo-SFAD is an advanced heterobifunctional photoreactive crosslinker that contains an amine-reactive sulfo-NHS ester on one end and a tetrafluorophenyl azide group on the other end. The fluorine substitutions on the phenyl ring prevent the photoactivated nitrene from reacting with the ring, thereby providing greater yields in capturing interacting proteins. The disulfide-containing cross-bridge allows cleavage of conjugated molecules by reduction with DTT.

2. Modify a purified bait protein by adding an aliquot of crosslinker to achieve a molar excess of 2–10 moles per mole of protein (protect from light). For NHS ester-containing reagents, react in 0.1 M sodium phosphate buffer at pH 7.2–7.4. Avoid using any other amine-containing buffer components, such as Tris or imidazole, which will interfere with the reaction.
3. React for 30–60 minutes in the dark (room temperature or 4°C).
4. Remove excess crosslinker from the modified bait protein by gel filtration or dialysis in the dark.

5. Add the modified bait protein to a sample containing potentially interacting prey proteins and incubate for 1 hour protected from light. The sample may be cells (for cell–surface interaction studies), cell lysate, or various extracts from cells, tissues, or biological fluids.
6. Expose the sample to UV light to crosslink and capture interacting proteins via the photoreactive group.
7. Recover interaction complexes by affinity chromatography. This is typically done using immobilized antibodies to the bait protein or using fusion tag binders, if the bait protein contains a fusion partner (e.g., immobilized glutathione for binding GST labeled bait proteins).
8. Analyze the purified complexes by SDS-PAGE, Western blotting, and mass spec.

3. Trifunctional Label Transfer Reagents

The earliest examples of label transfer reagents were cleavable heterobifunctional compounds that incorporated a phenyl azide group, which also had a phenolic modification on the ring. The phenolic hydroxyl activates the ring for substitution reactions to occur ortho or para to its position. These compounds thus can be radioiodinated using typical oxidation reagents such as chloramine-T or Iodobeads. Iodination of the crosslinker with ^{125}I prior to its use results in a radioactive label transfer reagent that can tag an unknown interacting protein with a radiolabel after cleavage of the crosslinker's spacer arm.

Subsequent designs of label transfer reagents used non-radioactive labels to avoid the safety and regulatory issues posed by ^{125}I. Fluorescent constituents designed into cleavable photoreactive crosslinkers make possible transfer of a fluorescent label to an unknown interacting protein. An example of this type of reagent that incorporates a coumarin group is SAED, which has been derivatized with an azido group on the aromatic ring. The reagent is non-fluorescent prior to exposure to UV light, but upon photoactivation and coupling to interacting proteins, it becomes highly fluorescent. The reagent also has a disulfide bond that can be reduced, resulting in cleavage of the crosslinked proteins and transfer of the label to the unknown interacting species. In this case, the fluorescently labeled interacting proteins can be followed in cells to determine the site of interactions or the fate of the proteins after interacting.

The most advanced type of bioconjugation reagent that is designed to study interacting proteins is a trifunctional label transfer reagent. These compounds are a special category of trifunctional crosslinkers (see Chapter 6), which possess two reactive groups and a third arm containing a label. Typically, one of the reactive groups is thermoreactive and can be used to label a bait protein, while the second reactive group is usually photoreactive for selective activation and coupling upon UV irradiation. The third arm of the reagent can be designed to have a fluorescent group for detection or terminate in a biotin group for both detection and purification (Figure 28.10).

An important feature of trifunctional label transfer reagents is the presence of a cleavable cross-bridge on the arm containing the thermoreactive group. After a bait protein has been conjugated with an interacting prey protein using this type of reagent, the complex then can be cleaved, which releases the interacting proteins and transfers the label over to the prey protein. The label can be used to detect prey proteins on a Western blot or to purify them from the sample solution. Unlike the heterobifunctional compounds discussed previously for studying protein interactions, label transfer reagents allow specific detection and purification of unknown interacting prey proteins for subsequent analysis.

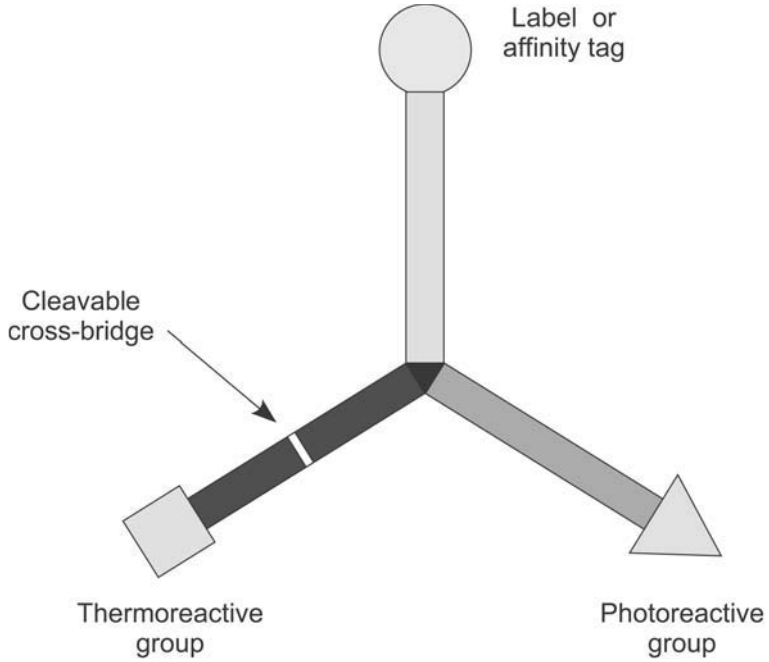


Figure 28.10 Trifunctional label transfer agents contain two arms with terminal reactive groups and a third arm with a label or affinity tag, such as a biotin group. One of the reactive groups typically is thermoreactive to couple with bait proteins, while the second reactive group usually is photoreactive. The thermoreactive arm has a cleavable cross-bridge to facilitate release of the captured protein and transfer of the label of affinity tag to it.

The following sections describe three types of label transfer reagents, which are all built with a biotin handle.

3.1. Sulfo-SBED

Sulfo-SBED is sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexanoamido] ethyl-1,3′-dithiopropionate), a trifunctional reagent containing an amine reactive sulfo-NHS ester on one arm, a photoreactive phenyl azide group on the second arm, and a biotin handle on its third arm (Figure 28.11; see Chapter 6, Section 2 for a general description and use). This compound has found great utility in capturing protein interactions and subsequently isolating them for analysis. A purified bait protein initially is labeled with Sulfo-SBED by its sulfo-NHS ester to form amide linkages through reaction with lysine or N-terminal amines. The modified bait then is incubated with a sample and biomolecules are allowed to interact with it. Finally, the sample is exposed to UV light and the photoreactive group is able to couple with nearby amine groups on any interacting proteins. The resultant complex can be purified, identified, or detected through specific binding to the biotin groups (Figures 28.12 and 28.13)

Sulfo-SBED is soluble in organic solvents, such as DMSO (125 mM), DMF (170 mM), and methanol (12 mM), or to a lesser degree in pure water (~5 mM). The solubility of Sulfo-SBED in

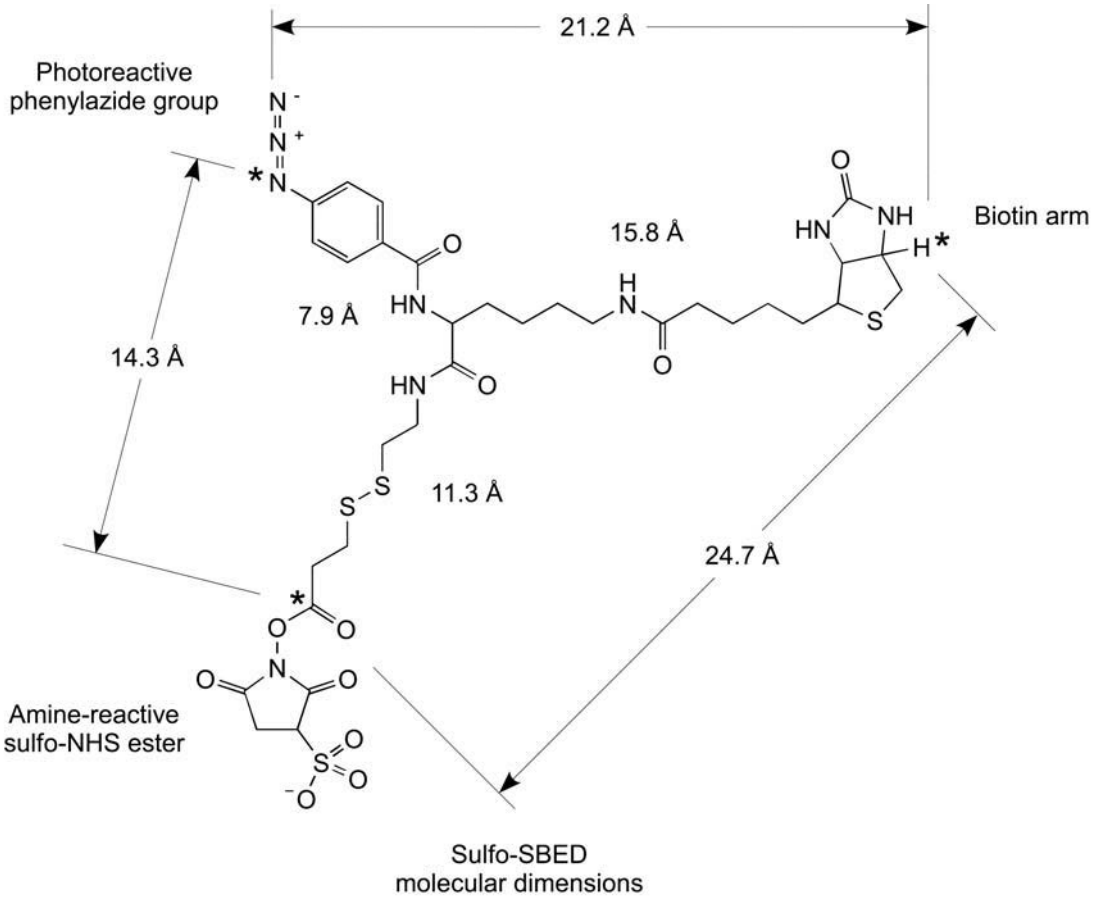


Figure 28.11 Sulfo-SBED is a label transfer agent that contains a water-soluble sulfo-NHS ester to label bait proteins and a phenyl azide group for photoreactive capture of a prey protein. The biotin label can be used for detection or isolation of protein–protein conjugates using (strept)avidin reagents. The stars indicate the atoms that were used to measure the indicated molecular dimensions.

buffered aqueous solutions may vary from about 0.1 mM to 3 mM (e.g., ~1 mM in 0.1 M PBS). However, to dissolve Sulfo-SBED at higher concentrations in a reaction buffer, first dissolve it in a water-miscible organic solvent such as DMSO or DMF and transfer an aliquot to the aqueous buffer solution. Use no more than about 1–10 percent of solvent in the final buffered reaction mixture to prevent buffer precipitation and minimize the denaturation of proteins.

Bait proteins modified with Sulfo-SBED may precipitate if the level of modification is too high, primarily due to the hydrophobic nature of the crosslinker and the biotin handle. To prevent precipitation or at least minimize it, adjust the molar excess of Sulfo-SBED over the bait protein to a level where the protein remains in solution. Some precipitation may be removed by centrifugation or filtration prior to use.

A derivative of Sulfo-SBED containing a thiol-reactive pyridyl disulfide group on its thermoreactive arm has been reported for modification of bait proteins containing a cysteine residue.

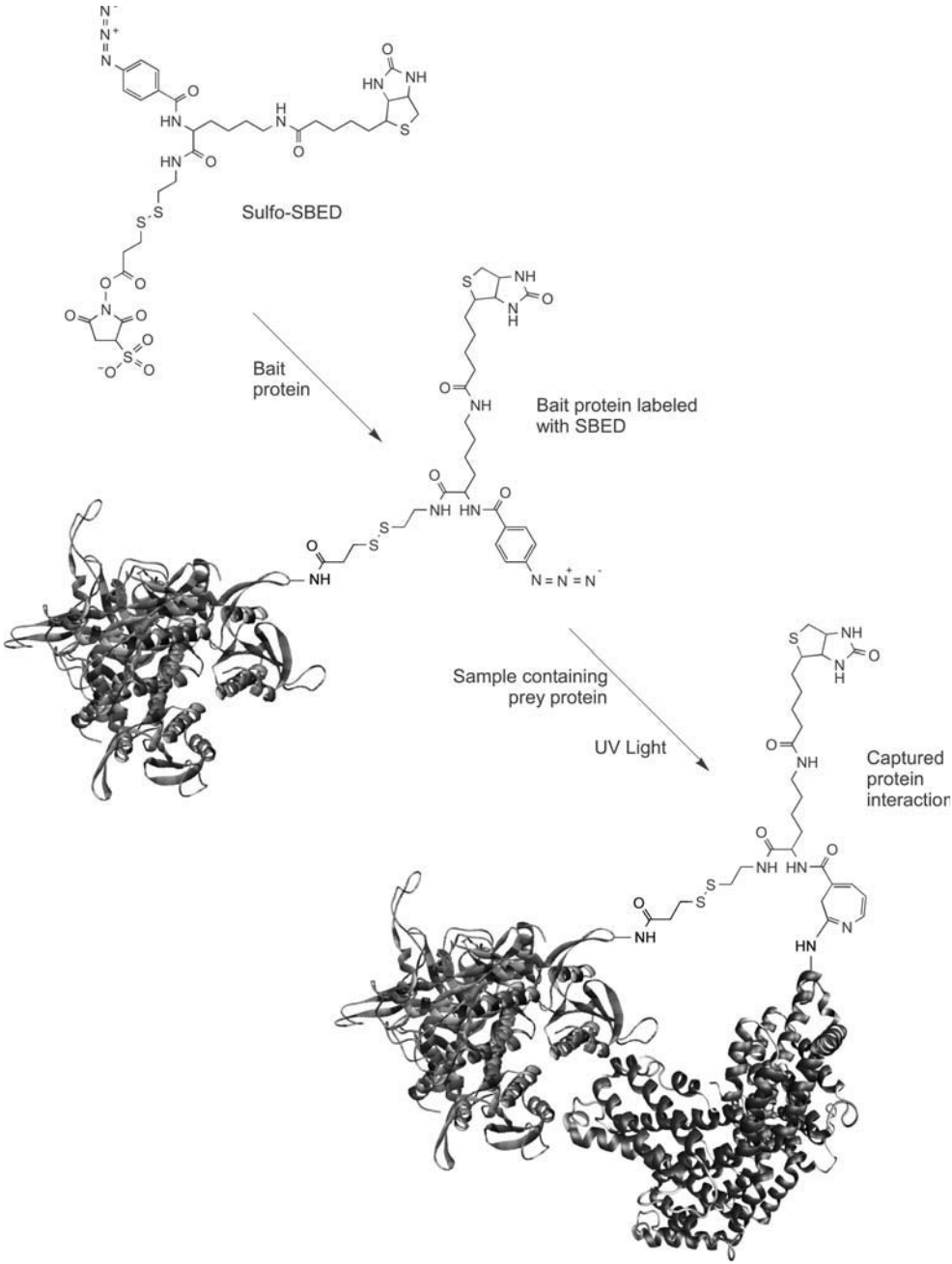


Figure 28.12 Sulfo-SBED first is used to label a bait protein through reaction of the sulfo-NHS ester with available amine groups on the protein, yielding an amide bond linkage. This labeled bait protein then is added to a sample containing proteins that potentially could interact with the bait. After an incubation period, the sample is exposed to UV light to photoactivate the phenyl azide group. This reaction causes any interacting prey proteins to be crosslinked with the bait protein, forming a complex containing a biotin affinity tag.

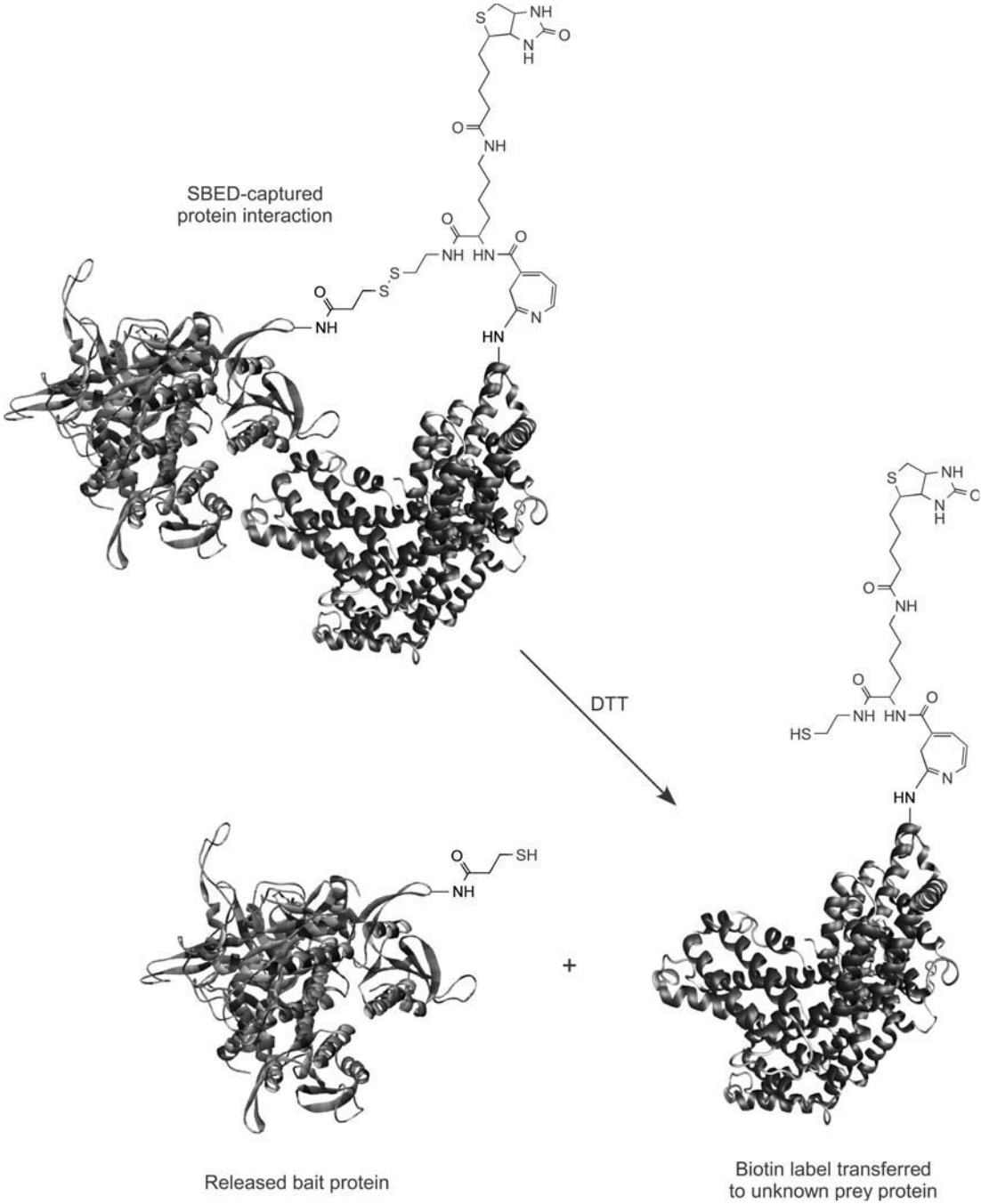


Figure 28.13 A sulfo-SBED-captured protein interaction can be released using DTT to cleave the disulfide within the cross-bridge leading to the bait protein. The result transfers the biotin label to the unknown interacting protein. The biotin tag thus allows the interacting protein to be detected or isolated using (strept)avidin reagents.

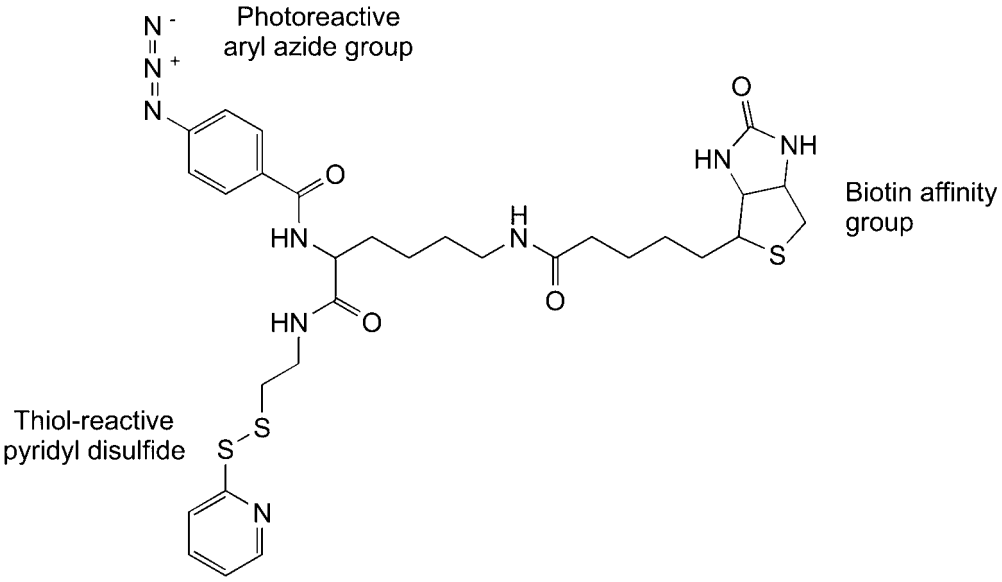


Figure 28.14 A trifunctional label transfer reagent containing a thiol-reactive pyridyl disulfide group, a photoreactive phenyl azide, and a biotin affinity tag. This compound can be used to label bait proteins through available thiol groups and capture interacting prey proteins by photoreactive conjugation.

Reaction of this compound with a thiol results in the formation of a reversible disulfide linkage. After capturing interacting proteins with this reagent, reduction of the disulfide transfers the biotin label to the unknown prey protein(s). This compound was prepared from Sulfo-SBED by reduction of the disulfide in its sulfo-NHS ester arm followed by reaction with 2,2′-dithiodipyridine to give the thiol-reactive compound, 1-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]-2-(2′-pyridyldithio)ethane (Figure 28.14) (Alley *et al.*, 2000; Ishmael *et al.*, 2001, 2002; Trakselis, 2005). This derivative has most of the features of Sulfo-SBED in terms of being a trifunctional label transfer agent containing biotin, but extends its application to sulfhydryl-containing bait proteins.

Sulfo-SBED has been used to investigate protein–protein interactions in the following applications: studying the bacteriophage T4 replisome (Ishmael *et al.*, 2003), cell–surface antigens of mycoplasma species bovine group 7 in binding and activation of plasminogen (Bower *et al.*, 2003), bacterium–host protein–carbohydrate interactions (Ilver *et al.*, 2003), transcription activator interactions with multiple SWI/SNF subunits (Neely *et al.*, 2002), binding of protein D/E to the surface of rat epidermal sperm (Tubbs *et al.*, 2002), for the study of the gp41–gp59 complex in bacteriophage T4 helicase (Ishmael *et al.*, 2002), identification of a region in alcohol dehydrogenase that binds to α-crystallin during chaperone action (Santhoshkumar and Sharma, 2002), regions of the mouse CD14 molecule required for toll-like receptor 2- and 4-mediated

activation of NF-kB (Muroi *et al*., 2002), active site residues of cyclophilin A that are important for signaling via CD147 (Yurchenko *et al*., 2002), mapping protein–protein interactions in the bacteriophage T4 DNA polymerase holoenzyme (Alley *et al*., 2000), import of adenovirus DNA involving the nuclear pole complex receptor CAN/Nup214 and histone H1 interactions (Trotman *et al*., 2001), insulin-like growth factor (IGF)-1 interaction with IgG-binding proteins (Horney *et al*., 2001), 3F3/2 anti-phospho-epitope antibody binding to the mitotically phosphorylated anaphase-promoting complex/cyclosome (Daum *et al*., 2000), SH3 binding sites of ZG29p in its interaction with amylase (Kleene *et al*., 2000), studying the proteasome activator PA28 in Hsp90-dependent protein refolding (Minami *et al*., 2000), investigating functional elements in α-crystallin (Sharma *et al*., 2000), *Helicobacter pylori* adhesin binding to fucosylated histo-blood group antigens (Ilver *et al*., 1998), identification of low abundance proteins by electrophoresis and MALDI-TOF MS (Bergstrom *et al*., 1998), molecular probes for muscarinic receptors (Jacobson *et al*., 1995), in the quantitation of triple-helix formation (Geselowitz and Neumann, 1995), studying prion protein interactions with its receptor (Santuccione *et al*., 2005), the activation of Hsp70 chaperones (Steel *et al*., 2004), effect of oxidized β_B3-crystallin peptide on lens β_L-crystallin and its interaction with β_B2-crystallin (Udupa and Sharma, 2005), the effect of oxidized β_B3-crystallin peptide on the thermal aggregation of bovine lens gamma-crystallins (Udupa and Sharma, 2005), for the mass spectrometric detection of affinity purified crosslinked peptides (Hurst *et al*., 2004), in mapping protein interfaces combined with MALDI-TOF and ESI-FTICR mass spectrometry (Sinz *et al*., 2005), studying the activation of the antioxidant enzyme 1-CYS peroxiredoxin and its requirement for glutathionylation mediated by binding with GST (Manevich *et al*., 2004), investigating the recruitment of HAT complexes by direct activator interactions with the ATM-related Tra1 subunit (Brown *et al*., 2001), the identification of annexin A2 heterotetramer as a receptor for the plasmin-induced signaling in human peripheral monocytes (Laumonnier *et al*., 2005), and in the role of the proteasome activator PA28 in the Hsp90-dependent protein refolding (Minami *et al*., 2000).

In addition to the use of Sulfo-SBED to capture unknown prey proteins interacting with labeled bait proteins, the reagent also may be used to study the interaction interfaces between two known proteins that specifically interact. Sinz *et al*. (2005) used this approach to identify the interaction surfaces between calmodulin and M13, which is a short peptide from skeletal muscle light chain kinase. In this application, calmodulin was labeled with Sulfo-SBED and allowed to interact with purified M13. After an incubation period, the solution was exposed to UV light and the two proteins were crosslinked together. Next, the conjugated proteins were proteolytically digested with trypsin and the resultant biotinylated peptides were enriched on a column of immobilized monomeric avidin (Thermo Fisher). The crosslinked peptides come from one peptide segment of calmodulin and one peptide segment of M13. Finally, these biotinylated and crosslinked peptides were identified using nano-HPLC separation into a nano-ESI-FTICRMS with software-facilitated deconvolution of the peptide identities. Such analysis provides insight into the interaction surfaces involved with the binding event between the two proteins.

The following protocol represents a suggested method that will work well for many proteins. It is a blend of protocols used in the literature and recommended by Thermo Fisher in the Sulfo-SBED instruction manual. Modifications to reaction conditions may be necessary in certain cases to maintain protein stability or solubility, depending on the properties of the particular bait protein being used.

Protocol

1. Dissolve a purified bait protein in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2, or a similar buffer at neutral pH, which doesn't contain any competing amines (i.e., avoid Tris or Imidazole). The bait protein may be at a concentration of anywhere from 0.1 mg/ml to 10 mg/ml. Prepare the solution in a dark tinted vial or wrap the vessel in foil to prevent light exposure when the crosslinker is added.
2. Prepare a solution of Sulfo-SBED in dry DMF or DMSO at a concentration of 40 µg/ml. Protect from light.
3. Add a quantity of the Sulfo-SBED solution to the bait protein solution so that a 1- to 5-fold molar excess of crosslinker over the bait protein results in the reaction mixture. Mix well. Using greater quantities of Sulfo-SBED to the bait protein may result in precipitation due to the hydrophobic nature of crosslinker. In addition, over modification of the bait protein with the crosslinker may block sites of protein interaction, thus preventing complex formation. As a practical example, Horney *et al.* (2001), used a 1:1 molar ratio of Sulfo-SBED to the bait protein IGF-1 with success.
4. React for 30 minutes at room temperature or 2 hours at 4°C. Continue to protect the solution from light.
5. Remove excess crosslinker and reaction by-products by dialysis or size exclusion chromatography. For small quantities of bait proteins, dialysis may be the better choice, because gel filtration columns often bind nonspecifically enough protein to make recoveries unacceptably low.
6. Add the labeled bait protein to a sample containing the putative interacting prey proteins. The quantity of bait protein to be added to a given sample should be within the same concentration level as the amount of prey proteins present. The optimal level of addition may have to be determined by varying the amount of bait protein concentrations in a number of sample solutions to decide which concentration results in the best interaction complexes being formed.
7. Incubate the sample mixture for at least 1 hour at room temperature or under conditions optimal for the interaction to be studied.
8. Expose the sample to UV light to photoactivate the aryl azide and cause the conjugation reaction to occur. For best results, use a UV lamp that irradiates in the range of 300–370 nm. Irradiate for 2–15 minutes, using a briefer exposure when using UV lamps of greater wattage.
9. Interaction complexes that were captured by the crosslinking reaction can be recovered or analyzed using the biotin groups. An immobilized streptavidin support, for instance, can be used to purify the conjugates away from other sample proteins. Alternatively, a streptavidin–HRP conjugate can be used to probe a Western blot after electrophoresis separation of the sample. Reduction of the conjugate may be done by cleavage of the disulfide bond with DTT. The typical addition of DTT to the electrophoresis sample buffer will facilitate disruption of the complexes and transfer of the biotin label to the interacting prey proteins. This reduction step also can be done using 50 mM DTT or 50 mM TCEP in aqueous solution to permit recovery of the biotinylated prey proteins on immobilized streptavidin. Alternatively, the use of an affinity support having milder elution characteristics for biotinylated proteins, such as immobilized monomeric avidin, would facilitate isolation of the biotinylated complexes or prey proteins under non-denaturing conditions.

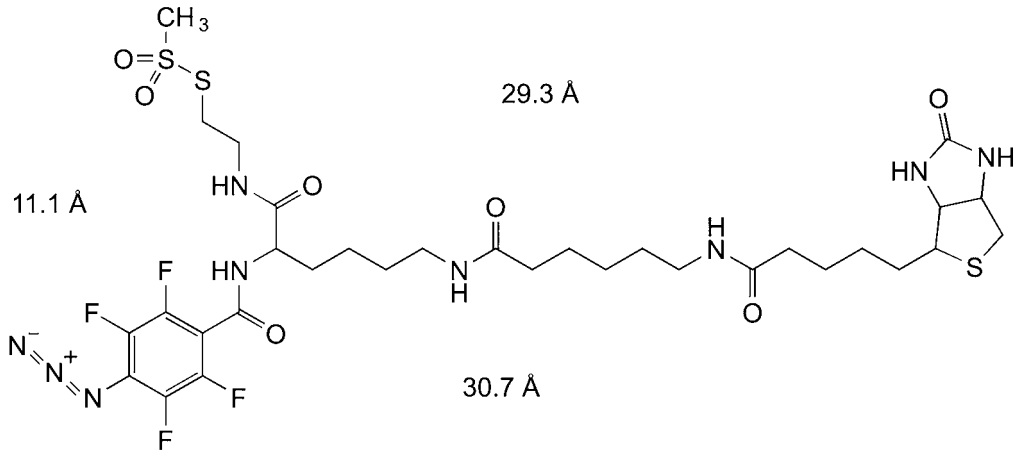
3.2. MTS-ATF-Biotin and MTS-ATF-LC-Biotin

MTS-ATF-Biotin is 2-[N2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-N6-(6-biotinamidocaproyl)-L-lysinyl]ethyl methanethiosulfonate and MTS-ATF-LC-Biotin is 2-{N2-[N6-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-6-aminocaproyl]-N6-(6-biotinamidocaproyl)-L-lysinylamido}]ethyl methanethiosulfonate. Both reagents are trifunctional crosslinkers similar in design to Sulfo-SBED discussed previously, but in addition to the biotin handle, they contain a thiol-reactive group and an enhanced photoreactive, perfluorinated phenyl azide group. The two reagents differ in the length of the cross-bridge in the photoreactive arm, with the LC version containing an extended aminocaproyl spacer. Relative to the spacing possible between the reactive groups on these compounds, the LC version therefore provides nearly twice the maximal molecular distance over its shorter analog (21.8 Å versus 11.1 Å) (Figure 28.15). Thus, interacting proteins may be captured either through use of a long or short crosslink, depending on the optimal distances between the proteins–or at least to the nearest thiol on the bait protein.

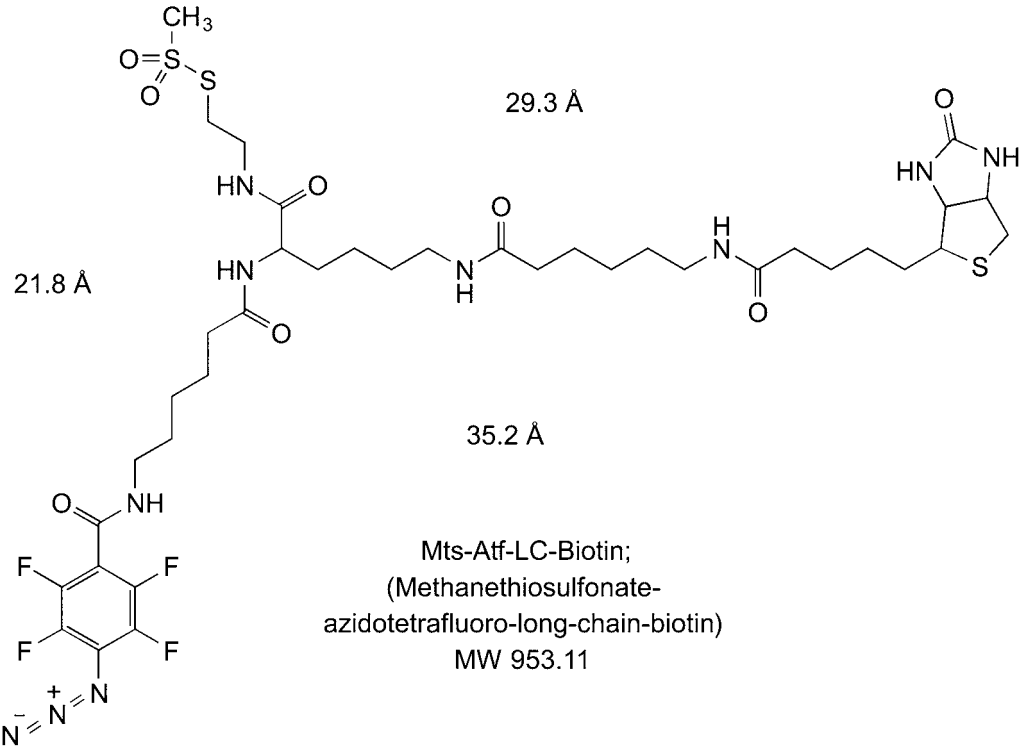
Both MTS-ATF-Biotin and MTS-ATF-LC-Biotin contain a methanethiolsulfonate group (MTS) on their thermoreactive arm, which is able to couple with the sulfhydryl on a cysteine residue. This reaction proceeds with loss of the methyl sulfonate leaving group (sulfinic acid) to produce a disulfide linkage (Figure 28.16). Unlike a pyridyl disulfide group, however, which also reacts with thiols to form disulfide linkages, the MTS group is unstable to hydrolysis in aqueous solution, especially if other strong nucleophiles are present. Therefore, most MTS compounds dissolved or brought into PBS buffer at physiological pH will hydrolyze with a half-life on the order of 10–15 minutes. However, they also have very rapid reactivities with thiols (Stauffer and Karlin, 1994; Holmgren *et al*., 1996; Liu *et al*., 1996). If the parent MTS reagent is fully water-soluble, the reaction with a thiol on a bait protein can take place with high yields in just a matter of minutes. However, both of these trifunctional label transfer compounds are hydrophobic, so their MTS reactivity in the aqueous phase may be somewhat slower than corresponding hydrophilic MTS reagents.

For use in studying protein interactions, these compounds first are reacted with a thiol on a purified bait protein to form a disulfide bond. Since the reagents are water-insoluble, they must be dissolved in an organic solvent such as DMF or DMSO and then an aliquot added to the bait protein in an aqueous buffer to initiate the reaction. Once modified, the biotinylated bait protein then is incubated with a sample containing potential interactive prey proteins. After an incubation period, initiating the photoreaction by exposure to UV irradiation captures the interacting proteins. Any interaction complexes thus formed can be isolated or detected using the biotin handle. In addition, the disulfide bond formed with the bait protein during the crosslinking reaction can be reduced to cleave the conjugates and transfer the biotin label to the unknown interacting prey proteins. This is a powerful way of labeling unknown interacting proteins for subsequent analysis.

Although MTS-ATF-Biotin and MTS-ATF-LC-Biotin are available commercially (Thermo Fisher and Toronto Research), they are relatively new and don't have the publications or applications backing up their use as has Sulfo-SBED. Therefore, the following protocol should be used as a suggested starting point to develop optimized methods for studying specific protein interactions. The bait protein to be modified with MTS-ATF-Biotin or MTS-ATF-LC-Biotin should be purified and contain at least one accessible thiol for reaction with the MTS group. Ellman's reagent (Chapter 1, Section 4.1) may be used to determine if a sulfhydryl is present and able to react with the crosslinkers. A free thiol also may be created by reduction of internal



Mts-Atf-Biotin;
(Methanethiosulfonate-
azidotetrafluoro-Biotin)
MW 839.95



Mts-Atf-LC-Biotin;
(Methanethiosulfonate-
azidotetrafluoro-long-chain-biotin)
MW 953.11

Figure 28.15 Two similar label transfer reagents containing a thiol-reactive methanethiolsulfonate group to label bait protein through available sulfhydryls, a tetrafluorophenyl azide group for high-efficiency photoreactive conjugation with interacting prey proteins, and a long biotin affinity tag.

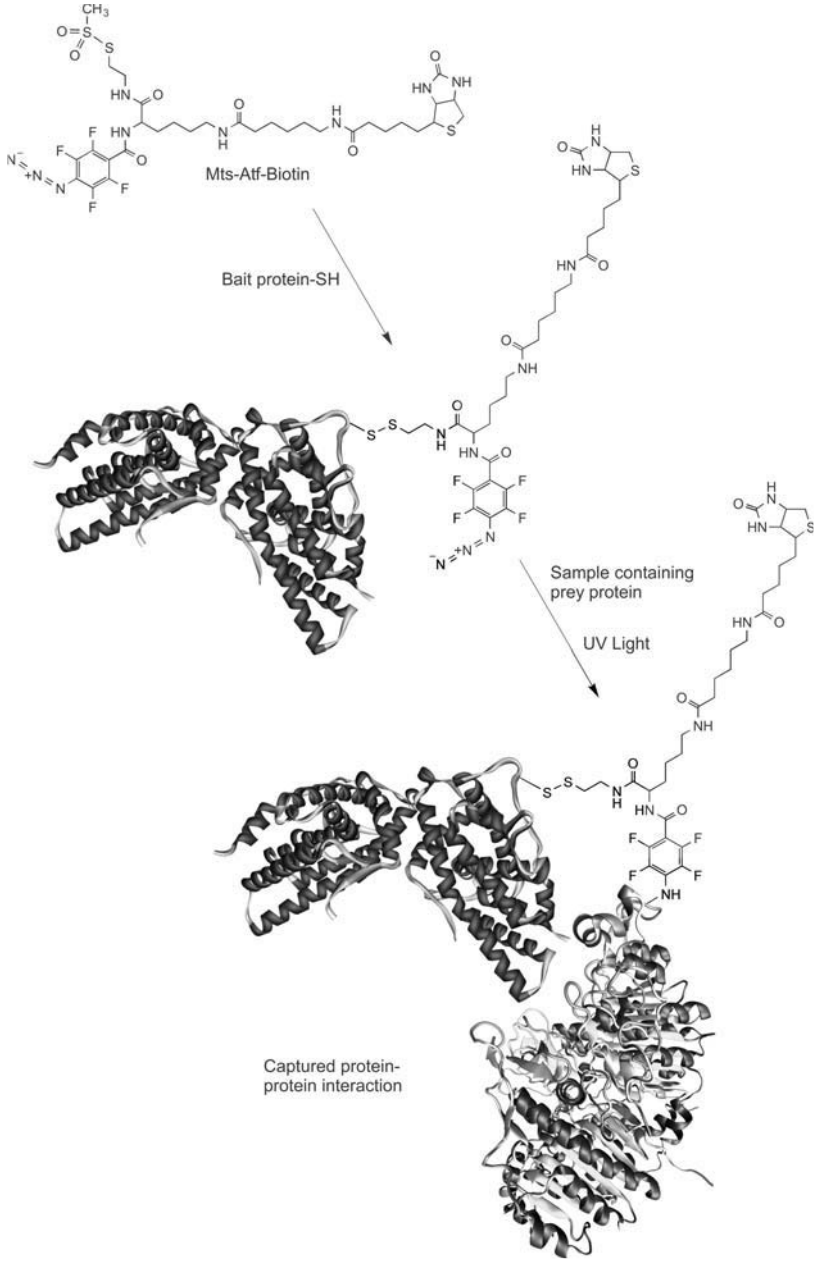


Figure 28.16 Mts-Atf-biotin can be used to modify a bait protein using an available thiol group to form a disulfide bond. The labeled bait protein then is allowed to interact with a sample containing proteins that potentially can interact with the bait. After an incubation period, the sample is exposed to UV light to photoactivate the tetrafluorophenyl azide group. This reaction causes any interacting prey proteins to be crosslinked with the bait protein, forming a complex containing a biotin affinity tag. Subsequent cleavage of the disulfide bond to the bait protein using DTT transfers the biotin group to the unknown interacting protein. The labeled prey protein then can be detected or isolated using (strept)avidin reagents.

disulfides or through the use of a thiolation reagent. However, adding thiols by chemical derivatization may not be advantageous, because this will create numerous surface modifications, which may have detrimental effects on protein–protein interactions.

Protocol

1. Dissolve a thiol-containing bait protein in 10 mM HEPES, 0.15 M NaCl, pH 7.5 or in 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2. Other buffers around physiological pH will work well; however avoid the presence of reducing agents containing thiols (i.e., DTT or 2-mercaptoethanol). Typical concentrations that will work with the suggested protocol range from 100 μg/ml to 10 mg/ml, but higher concentrations are preferred to give high reaction yields.
2. Dissolve MTS-ATF-Biotin or MTS-ATF-LC-Biotin in DMF or DMSO at a concentration of at least 10 mg/ml. Protect from light.
3. Add a quantity of the crosslinker solution to the bait protein solution to provide a 1–5 molar excess of the crosslinker over the quantity of protein to be modified. The addition of the organic solution containing the crosslinker should be done so that the final reaction contains no more than 10 percent organic solvent. Even lower concentrations of solvent may be required for certain sensitive proteins.
4. React for at least 30 minutes at room temperature or 1 hour at 4°C. Continue to protect the solution from light.
5. Remove excess crosslinker and reaction by-products by dialysis or size exclusion chromatography.
6. Add the labeled bait protein to a sample containing the putative interacting prey proteins. The quantity of bait protein to be added to a given sample should be within the same concentration level as the amount of prey proteins present. The optimal level of addition may have to be determined by varying the amount of bait protein concentrations in a number of sample solutions to decide which concentration results in the best interaction complexes being formed.
7. Incubate the sample mixture for at least 1 hour at room temperature or under conditions optimal for the interaction to be studied.
8. Expose the sample to UV light to photoactivate the aryl azide and cause the conjugation reaction to occur. For best results, use a UV lamp that irradiates in the range of 300–370 nm. Irradiate for 2–15 minutes, using briefer exposures when using UV lamps of higher wattage.
9. Interaction complexes that are captured by the crosslinking reaction can be recovered or analyzed using the biotin groups. For instance, immobilized streptavidin can be used to purify the conjugates or a streptavidin–HRP conjugate can be used to probe a Western blot after electrophoresis separation of the sample. Reduction of conjugates may be done by cleavage with DTT of the disulfide bond linking the bait protein. This can be done using 50 mM DTT or 50 mM TCEP in aqueous solution to permit recovery of the biotinylated prey proteins on immobilized streptavidin. Alternatively, the use of an affinity support having milder elution characteristics for biotinylated proteins, such as immobilized monomeric avidin, would facilitate isolation of the biotinylated complexes or prey proteins under non-denaturing conditions.

4. Metal Chelates in the Study of Protein Interactions

Certain bifunctional metal chelating agents have been used to investigate protein interactions by virtue of their ability to generate reactive oxygen species that affects protein structure in the immediate vicinity of their modification site. The following sections discuss two applications of such chelate labels, one of which cleaves peptide bonds while the other one causes covalent crosslinks to occur between interacting protein structures.

4.1. FeBABE for Protein Mapping Studies

Transition metals such as iron can catalyze oxidation reactions in aqueous solution, which are known to cause modification of amino acid side chains and damage to polypeptide backbones (see Chapter 1, Section 1.1; Halliwell and Gutteridge, 1984; Kim *et al*., 1985; Tabor and Richardson, 1987). These reactions can oxidize thiols, create aldehydes and other carbonyls on certain amino acids, and even cleave peptide bonds. The purposeful use of metal-catalyzed oxidation in the study of protein interactions has been done to map interaction surfaces or identify which regions of biomolecules are in contact during specific affinity binding events.

Hydroxyl radical "footprinting", as the technique is called, can produce a picture of where ligand binding sites are located or map the interaction surfaces of protein complexes. An early example of this application was used to map the binding site of protein–DNA interactions (Tullius and Dombroski, 1986; Latham and Cech, 1989). Hydroxyl radicals are generated by an EDTA chelated iron(II) complex using a redox reaction involving hydrogen peroxide and ascorbic acid. In this case, the EDTA chelate is merely added to the solution of the interacting protein and DNA. Nonselective cleavage of the DNA strand (and protein) then occurs everywhere except at the region in which the interaction takes place, thus leaving a "footprint" of the interacting partners.

A second iteration of this reagent involved the attachment of affinity ligands to the EDTA chelator group to direct the hydroxyl radical generator to specific binding pockets on proteins and receptors. Hoyer *et al*. (1990) created a biotin-EDTA derivative that was able to bind to the biotin binding pocket on streptavidin and cause peptide cleavage in the region immediately surrounding the binding site. Similarly, Schepartz and Cuenoud (1990) formed a trifluoroperazine-based affinity reagent containing an EDTA group to map the binding site of the calcium binding protein calmodulin. Once affinity reagents of this sort bind to their receptor protein, hydroxyl radical footprinting can identify exactly where in the protein's three-dimensional structure the interaction took place.

Rana and Meares (1990b, 1991a, b) created a more versatile footprinting reagent by synthesizing a bifunctional metal chelating agent with an EDTA group on one end and a bromoacetyl group on the other end. This compound, Fe(III) (S)-1-(*p*-bromoacetamido-benzyl)ethylene diamine tetraacetic acid (or FeBABE, pronounced "iron-babe"), resulted in a universal modification reagent for protein interaction footprinting, which could be used to study interacting protein complexes or other biomolecules (Figure 28.17). The bromoacetyl group of FeBABE can react with a thiol to form a thioether bond (Figure 28.18). Thus, proteins or other molecules containing a thiol can be covalently modified with the FeBABE reagent forming a hydroxyl radical probe to footprint interacting domains. Such modified bait proteins are termed "artificial proteases", because of their ability to chemically cleave peptide bonds.

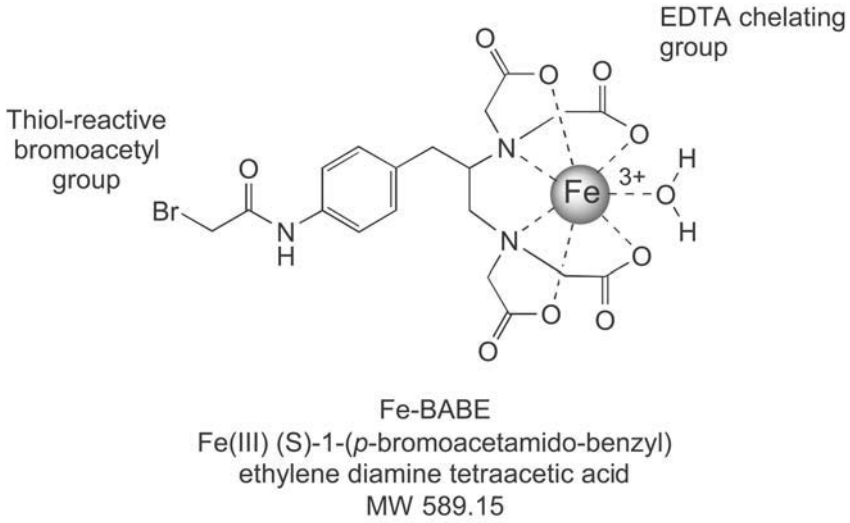


Figure 28.17 FeBABE is a bifunctional chelating agent containing an EDTA group on one side and a thiol-reactive bromoacetyl group on the other end. The EDTA group is coordinated with an iron ion.

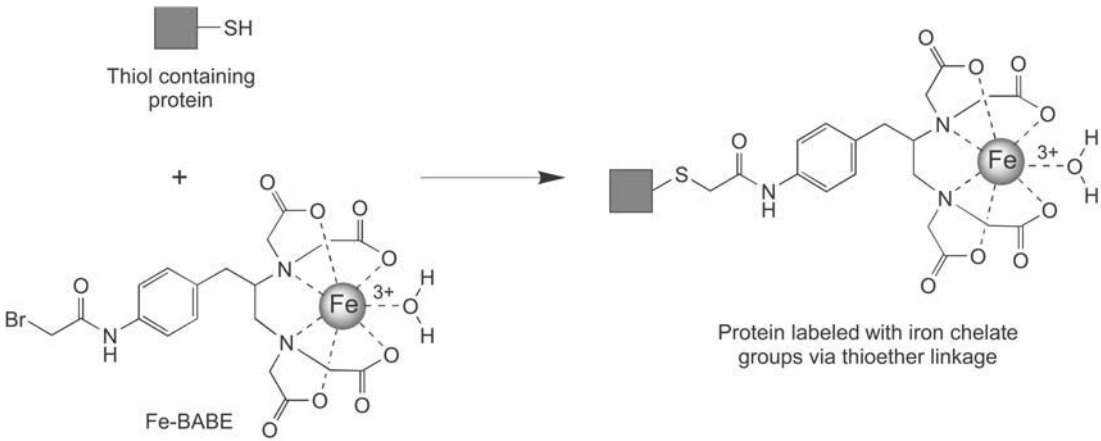


Figure 28.18 FeBABE can be coupled to an available thiol group on a bait protein using the bromoacetyl reactive group to form a thioether linkage.

The reactions involved in the cleavage of biological molecules by FeBABE and similar iron-EDTA complexes have been theorized to involve two possible processes. Rana and Meares (1991) speculated that the reaction proceeds by the coordination of peroxo oxygen to the iron chelate group. They also found that O_2 might participate in this type of reaction, as evidenced by the successful incorporation of ^{18}O into the carboxyl terminus of cleaved peptide chains of human carbonic anhydrase. This peroxo-metal complex may facilitate subsequent nucleophilic attack of oxygen on the carbonyl carbon of a peptide bond, which then results in cleavage of the peptide bond through oxygen atom transfer to the carbonyl carbon, forming a C-terminal carboxylate. Figure 28.19 schematically shows part of this reaction sequence.

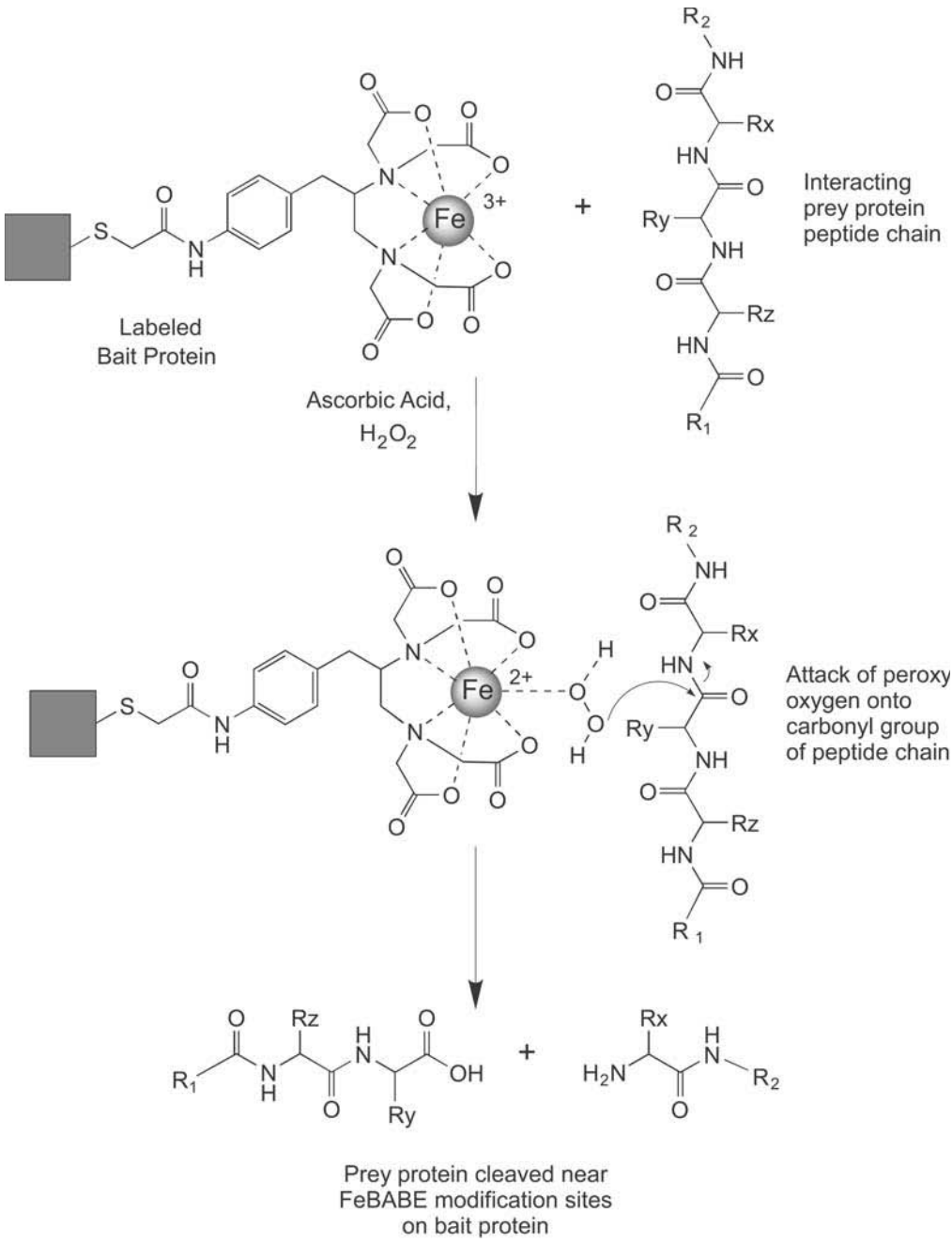


Figure 28.19 The cleavage reaction of FeBABE involves a catalytic process using peroxide and ascorbate to form reactive oxygen species. Any protein structure in the immediate vicinity of the FeBABE label on the bait protein will undergo peptide bond cleavage.

Another proposed mechanism involves the well-known Fenton reaction, which is catalyzed effectively by Fe^{2+}-EDTA complexes. In this process, the ascorbate functions by reducing the FeBABE Fe^{3+} complex to Fe^{2+}, producing the ascorbate radical ($^{\bullet}Asc^{-}$). The reduced iron then can form hydroxyl radicals through the Fenton reaction, which re-oxidizes the iron to the Fe^{3+} state. As additional Fe^{3+} is formed, ascorbate then regenerates the active Fe^{2+} chelate by reduction, resulting in the formation of the ascorbate radical. The entire process thus is catalytic and may occur numerous times at the site of one FeBABE modification, potentially resulting in a number of peptide bond fragmentations. Notice also that the FeBABE reagent can be used in an initial EDTA-Fe^{3+} form or in the reduced EDTA-Fe^{2+} form, as addition of the peroxide/ascorbate activators can catalyze hydroxyl radical formation from either metal oxidation state.

$$\text{EDTA-Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{EDTA-Fe}^{3+} + {}^{\bullet}\text{OH} + \text{OH}^{-} \quad \text{Fenton reaction}$$

$$\text{EDTA-Fe}^{3} + \text{ascorbate} \rightarrow \text{EDTA-Fe}^{2+} + {}^{\bullet}\text{Asc}^{-} + \text{H}^{+} \quad \text{Regeneration with ascorbate}$$

The FeBABE modifying group is approximately 12 Å in length. Peptide bond cleavage can occur anywhere on a protein surface that is within molecular distance of the iron chelate modifying group. Cleavage probably doesn't occur at distant sites, because the hydroxyl radical is extremely reactive and will quickly react with the neighboring peptide structure or be quenched by ascorbate in solution. An interacting protein that is within the molecular distance of FeBABE's reach therefore is susceptible to peptide bond cleavage.

In use, a bait protein labeled with FeBABE is bound to an interacting prey protein and the peroxide/ascorbate cleavage reagents added to initiate the protein mapping reaction. After the cleavage process is complete, the peptide fragments on each protein are analyzed to gleam information about the interaction surfaces, which will be left relatively unaffected by the reaction. Obviously, to utilize this technique the two interacting proteins must be highly pure and their amino acid sequences known.

Typically, a bait protein is labeled with FeBABE at any available cysteine thiol group. If no free thiols are available, disulfides may be reduced to create sulfhydryls or a thiolation reagent may be used to add them to the protein surface (see Chapter 1, Section 4.1). Care should be taken when adding thiols through thiolation, however, because too high a substitution level could affect the ability of the bait protein to bind to the prey protein. One to three thiol substitutions on the surface of a bait protein should be sufficient to modify it with FeBABE and study protein interactions. A third option for adding a thiol is to change recombinantly one amino acid in the bait protein's sequence to a cysteine. Site directed mutagenesis can create a thiol at a known region of the protein's surface, thus permitting the study of protein interactions through rational design of FeBABE modifications.

In order to facilitate analysis of FeBABE produced fragments, the prey protein or biomolecule is labeled at one end with a tag that can be detected after electrophoresis, usually in a transfer blot. The tag can be a fusion tag, such as 6× His, or any other group that can be targeted with an antibody and detected. Alternatively, radiolabels and fluorescent labels have been used with prey molecules, including the use of end-labeled DNA to study where DNA binding proteins dock onto the oligonucleotide sequence.

The fragments formed by FeBABE fragmentation are analyzed by comparing them to enzymatic or chemical cleavage patterns observed by treatment on the same prey protein. Since the cleaved prey protein is detected by its end-labeled tag, the only fragments detected are those

that extend from the end labeled terminal to the point of cleavage. Comparison with known fragmentation patterns using protease digestion or chemical cleavage provides information as to the approximate size of the FeBABE fragment and its point of cleavage.

The following protocol assumes that the user has at least two pure proteins (or biomolecules) of known sequences, which are able to interact specifically in solution. One of the two proteins (the prey) is end labeled with a fusion tag or another detectable component and the other protein contains at least one thiol group. All buffers and reagents used in this protocol should be of high purity and contain a very low metal content to prevent nonspecific cleavage reactions.

Protocol

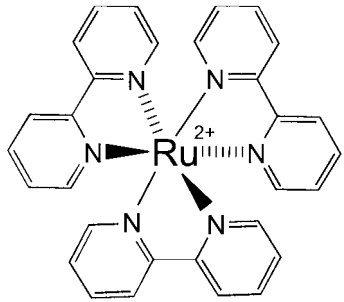
This protocol is based on the procedure of Rana and Meares (1991) and the instruction booklet provided by Thermo Fisher for use with the FeBABE kit.

1. Dissolve the bait protein to be modified with the FeBABE reagent in 50 mM MOPS, 100 mM NaCl, 1 mM EDTA, 5 percent glycerol, pH 8.2 (coupling buffer). The protein first may be treated by dialysis with EDTA to remove residual metals (30 mM MOPS, 4 mM EDTA, pH 8.2), and then dialyzed into the final coupling buffer for the reaction with FeBABE.
2. Dissolve FeBABE in DMSO at a concentration of 5 mg/ml (8.5 nmol/μl) and add an aliquot of this solution to the thiol-containing bait protein solution to obtain a 2- to 20-fold molar excess of the reagent over the protein concentration. Protect the FeBABE solution and the reaction mixture from light, as exposure to light will degrade the bromoacetyl reactive group. The optimal molar excess of FeBABE over the bait protein may have to be determined by experimentation. Since thiols are typically limited in number in most proteins, an excess of FeBABE reagent will not result in more labels than the total number of thiols present, provided the suggested reaction conditions are maintained.
3. Incubate the reaction mixture at room temperature or 37°C for 1 hour.
4. Purify the modified bait protein from excess reactants by desalting or dialysis. During this process buffer exchange the labeled bait protein into 50 mM MOPS, 120 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 percent glycerol, pH 8.0. Components may be added or removed from this buffer to promote the protein interaction being studied as long as they don't interfere with the subsequent FeBABE cleavage reaction (i.e., they don't contain transition metals like iron or copper, oxidants, or reducing agents).
5. Dissolve or buffer exchange the prey protein into 50 mM MOPS, 120 mM NaCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 10 percent glycerol, pH 8.0.
6. Mix the labeled bait protein with the prey protein at equal molar ratios and incubate for 30 minutes at room temperature to create protein interaction complexes.
7. Initiate the cleavage reaction by the addition of an 8-fold molar excess of ascorbate (over the concentration of protein) followed by an 8-fold molar excess of H_2O_2· Mix well.
8. React for 10–30 seconds at room temperature.
9. Quench the cleavage reaction by the addition of an equal volume of SDS electrophoresis sample buffer containing up to 40 percent glycerol. The SDS will denature the protein interaction and glycerol acts as a free radical scavenger, thus effectively quenching the reaction.
10. Analyze the cleaved proteins by SDS gel electrophoresis and Western blotting, followed by specific detection of the prey protein label. Comparison of prey protein fragments formed by chemical and enzymatic digestion can yield information as to the site of the resultant FeBABE-mediated cleavage.

4.2. Ru(II)bpy$_3^{2+}$ for Light-Triggered, Zero-Length Crosslinking of Protein Interactions

In many instances within cells or on cell membrane surfaces, large protein complexes are formed containing many different proteins specifically interacting to effect biological processes. Protein machines of this type are difficult to capture and study using traditional crosslinking agents, because the resultant reactions may link some of the proteins, but not all of them in the complex (Friedrichson and Kurzchalia, 1998; Simons *et al.*, 1999). In addition, covalent modification of many nucleophilic groups in a protein by the electrophilic reactive groups of standard crosslinking reagents can decorate the surface of proteins with these compounds and affect nonspecific protein interactions.

Fancy and Kodadek (1999) developed a novel protein crosslinking process using a metal chelating reagent that can be activated by visible light and effect zero-length crosslinks between proteins. Photo-induced crosslinking of unmodified proteins (PICUP) can capture protein interactions without the use of modifying agents such as organic crosslinkers or fusion tags, thus maintaining the interacting proteins in their native structure and conformation. The reaction is mediated by the chelate ruthenium(II) tris(bipyridine) (Ru(II)bpy$_3^{2+}$; pronounced "rūbippy") in a two-step reaction involving light absorption by the bipyridine groups followed by energy transfer and excitation of the Ru(II) metal to an excited state intermediate. Subsequent reaction of the excited state Ru(II)bpy$_3^{2+}$ with persulfate anion results in the formation of a sulfate radical and Ru(III). The Ru(III) group then is postulated to undergo a one-electron transfer to tyrosine side chain phenolic groups in proteins, resulting in the formation of a tyrosine radical. This highly reactive intermediate rapidly can react with neighboring nucleophilic groups in proteins or with another nearby tyrosine phenolic group, resulting in the formation of tyrosine dimers or a tyrosine heteroatom arene linkage with nearby nucleophilic groups. Either route of reaction can result in the formation of covalent bonds between protein amino acids. If the linkages are formed between interacting proteins, then these complexes are captured for subsequent analysis. The proposed reactions of Ru(II)bpy$_3^{2+}$ in the light-mediated zero-length crosslinking of interacting proteins are shown in Figure 28.21.



Ruthenium(II) tris(bipyridine) chelate

Ru(II)bpy$_3^{2+}$

MW 569.62

Figure 28.20 The structure of Ru(II)pby$_3^{2+}$ a chelated ruthenium ion coordinated with three bipyridine groups.

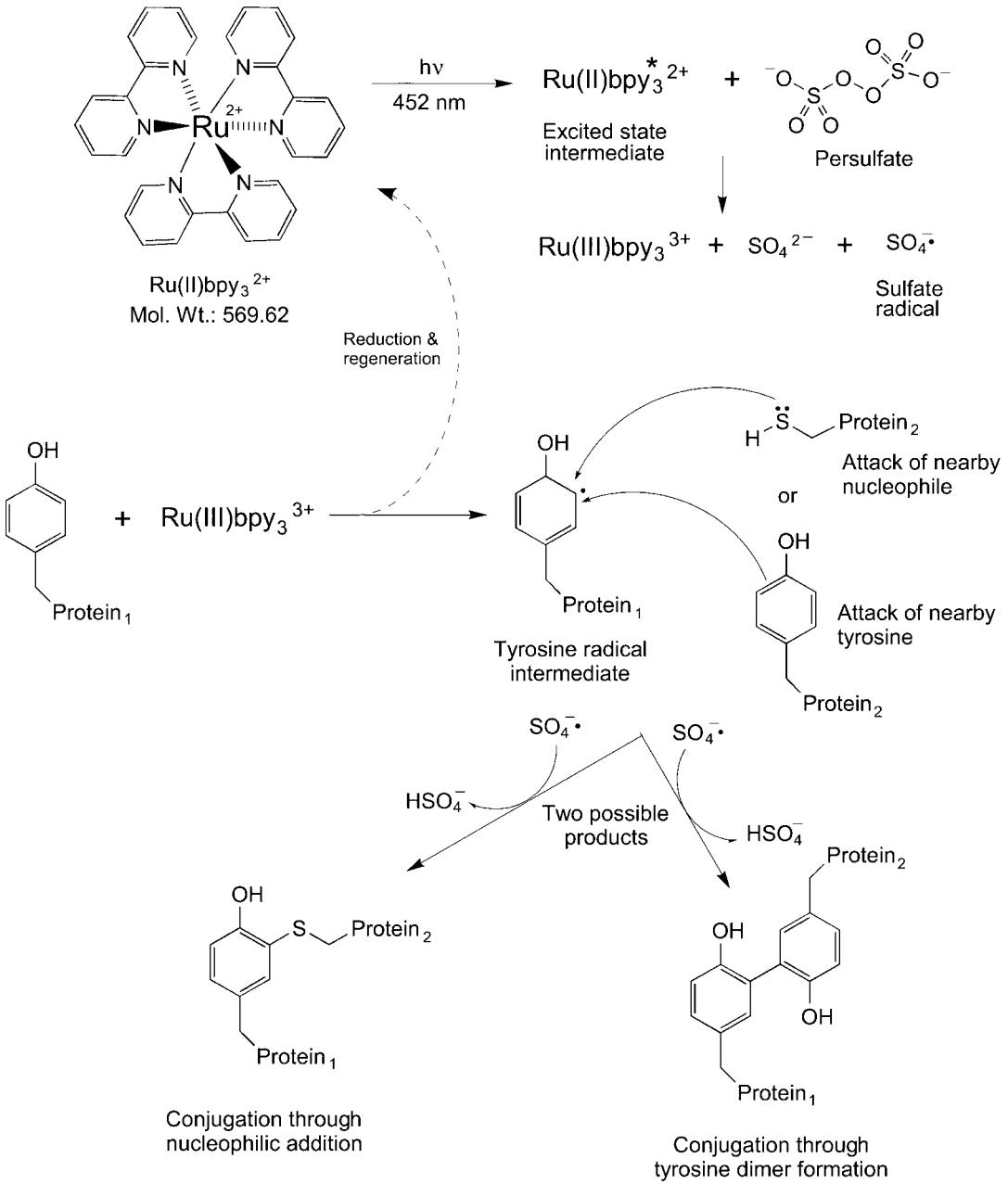


Figure 28.21 The reactions of Ru(II)pby$_3^{2+}$ are catalyzed by light at 452 nm that begins by forming an excited state intermediate. In the presence of persulfate, a sulfate radical is formed concomitant with the oxidative product Ru(III)bpy$_3^{3+}$. This form of the chelate is able to catalyze the formation of a radical on a tyrosine phenolic ring that can react along with the sulfate radical either with a nucleophile, such as a cysteine thiol, or with another tyrosine side chain to form a covalent linkage. The result of this reaction cascade is to cause protein crosslinks to form when a sample containing these components is irradiated with light.

The following protocol successfully was used to study the interactions of the 180-amino acid C-terminal domain of yeast TATA box binding protein (TBP), the Gal80 protein, and the Gal4 activation domain of the associated transcription factor complex (Fancy and Kodadek, 1999). It also was used for the study of the amyloid β-protein (αβ) assembly (Bitan *et al*., 2003), to characterize the tRNA-specific adenosine deaminase (tadA) from *E. coli* (Wolf *et al*., 2002) and to understand the nature of the interaction between MIP-1a and proteoglycans (Ottersbach and Graham, 2001). The reaction is very efficient and typically can result in conjugation yields of 60 percent or better between interacting proteins. In fact, the conjugation efficiency may yield crosslinks between proteins that are not specifically interacting. To limit the degree of extraneous conjugation, the addition of a nucleophile in the reaction medium (e.g., histidine) can help to restrict crosslinks only to proteins that are in close contact, as in those that are forming interaction complexes at the time.

Protocol

1. Adjust the protein sample concentration to be between 0.01 μM and 20 μM by dilution in 20 μl of reaction buffer: 15 mM sodium phosphate, 150 mM NaCl, 0.125 mM $Ru(bpy)_3Cl_2$ (pH 7.5). The protein sample should not contain any components that are easily oxidized by the reaction, such as thiol reducing agents (e.g., DTT). The addition of histidine to the reaction buffer (7.5 mM) can be done to modulate the crosslinking reaction and limit the degree of protein conjugation between non-interacting proteins. The presence of other nucleophiles also will inhibit the crosslinking reaction, such as Tris buffer or DTT.
2. Add an aliquot of ammonium persulfate (APS) to make a final concentration of 2.5 mM. Protect from light until ready to start the reaction.
3. Illuminate the sample in a small open tube or the well of a microplate by placing a white light source approximately 5–50 cm from the sample. If a weak light source is used, such as a flashlight, move the light source closer to the sample, but if a strong photoflood lamp is used, then use the greater distance measurement to avoid heating the sample. Illuminate the sample for 0.5–30 seconds, depending again on the intensity of the light source.
4. Immediately after irradiation, stop the reaction by the addition of 7 μl of 4 × SDS electrophoresis loading buffer or the equivalent (with a high concentration of reducing agent present): 0.2 M Tris, 8 percent SDS, 2.88 M β-mercaptoethanol, 40 percent glycerol, 0.4 percent xylene cyanol, 0.4 percent bromophenol blue. Heat the sample at 95°C for 5 minutes and analyze the complexes formed by electrophoresis.

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