

## 5.1 Introduction to Crosslinking Reagents

Bifunctional “crosslinking” reagents contain two reactive groups, thereby providing a means of covalently linking two target groups. The reactive groups in a chemical crosslinking reagent (Section 5.2) typically belong to the classes of functional groups — including succinimidyl esters, maleimides and iodoacetamides — described in Chapter 1, Chapter 2 and Chapter 3. In contrast, one of the reactive groups in each of our photoreactive crosslinking reagents (Section 5.3) requires light activation before reacting with a target group. Crosslinking of a biopolymer (such as an antibody, enzyme, avidin or nucleic acid) to a low molecular weight molecule (such as a drug, toxin, peptide or oligonucleotide) or to another biopolymer yields a stable heteroconjugate. This bioconjugate can serve as a detection reagent in a wide variety of research and diagnostic assays (Section 6.1) or as an immunogen designed to elicit antibody production. Crosslinking reagents are also useful for probing the spatial relationships and interactions within and between biomolecules.

In homobifunctional crosslinking reagents, the reactive groups are identical. These reagents couple like functional groups — typically two thiols, two amines, two acids or two alcohols — and are predominantly used to form intramolecular crosslinks. When used to conjugate two different biomolecules, for example an enzyme to an antibody, these relatively nonspecific reagents tend to yield high molecular weight aggregates.

In heterobifunctional crosslinking reagents (Table 5.1), the reactive groups have dissimilar chemistry, allowing the formation of crosslinks between unlike functional groups (Figure 5.1). As with homobifunctional crosslinking reagents, heterobifunctional crosslinking reagents can still form multiple intermolecular crosslinks to yield high molecular weight aggregates, but conjugations that use these reagents can be more easily controlled so as to optimize the stoichiometry of the target molecules. Thus, heterobifunctional crosslinking reagents are very useful for preparing conjugates between two different biomolecules.

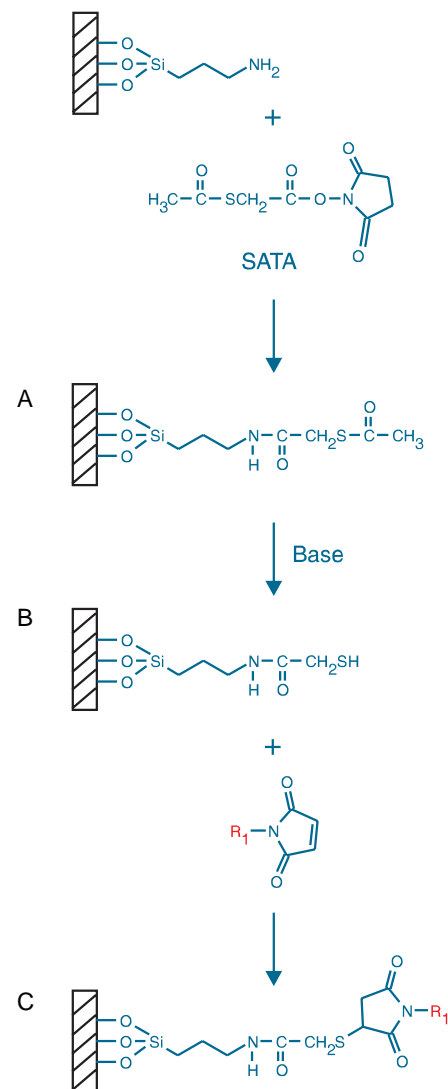
The photoreactive crosslinking reagents are a special subset of the heterobifunctional crosslinking reagents. Upon UV illumination, these reagents react with nucleophiles or form C-H insertion products (Figure 5.8, Figure 5.9, Figure 5.10).

An additional variation is the “zero-length” crosslinking reagent — a reagent that forms a chemical bond between two groups without itself being incorporated into the product (Figure 3.22). The water-soluble carbodiimide EDAC (E-2247, Section 5.2), which is used to couple carboxylic acids to amines, is an example of a zero-length crosslinking reagent.

**Table 5.1** Molecular Probes' heterobifunctional crosslinkers.

Cat #	Crosslinker	Reactivity			
		Photo-reactive *	Aldehyde or Ketone	Amine (R <sub>1</sub> -NH <sub>2</sub> )	Thiol (R <sub>1</sub> -SH)
S-1553	Succinimidyl acetylthioacetate (SATA)			✓	✓
S-1534	succinimidyl <i>trans</i> -4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC)			✓	✓
S-1531	succinimidyl 3-(2-pyridyldithio)propionate (SPDP)			✓	✓
P-6317	<i>N</i> -((2-pyridyldithio)ethyl)-4-azidosalicylamide (PEAS; AET)	✓			✓
A-2522	4-azido-2,3,5,6-tetrafluorobenzoic acid, succinimidyl ester (ATFB, SE)	✓		✓	
A-10661	4-azido-2,3,5,6-tetrafluorobenzoic acid, STP ester, sodium salt (ATFB, STP ester)	✓		✓	
A-10662	4-azido-2,3,5,6-tetrafluorobenzyl amine, hydrochloride	✓	✓		
B-1526	benzophenone-4-isothiocyanate	✓		✓	
B-1508	benzophenone-4-maleimide	✓			✓
B-1577	4-benzoylbenzoic acid, succinimidyl ester	✓		✓	

\* Reacts nonspecifically with available nucleophiles upon UV illumination.

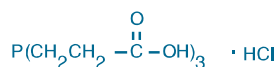


**Figure 5.1** Schematic illustration of the heterobifunctional crosslinker succinimidyl acetylthioacetate (SATA, S-1553): A) attachment to an aminosilane-modified surface, B) deprotection with base and C) reaction with a sulfhydryl-reactive biomolecule.

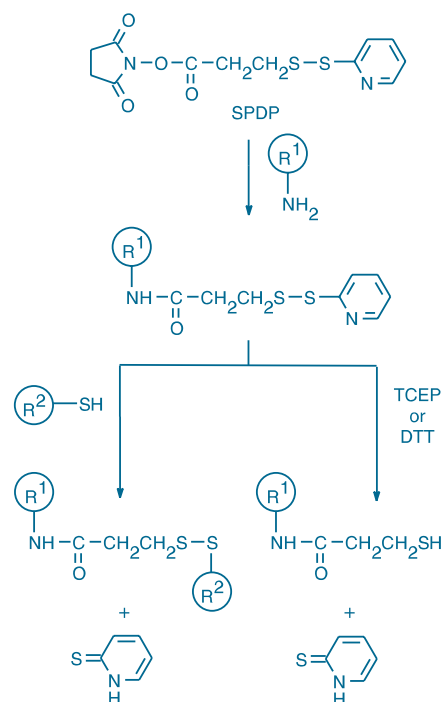
*Our Zenon Antibody Labeling Kits (Section 7.2) make the preparation of R-phycoerythrin, allophycocyanin, alkaline phosphatase and horseradish peroxidase conjugates of antibodies far easier and higher yield than any chemical coupling methods.*

A noncovalent interaction between two molecules that has very slow dissociation kinetics can also function as a crosslink. For example, reactive derivatives of phospholipids can be used to link the liposomes or cell membranes in which they are incorporated to antibodies or enzymes. Biotinylation and haptenylation reagents (Chapter 4) can also be thought of as heterobifunctional crosslinking reagents because they comprise a chemically reac-

tive group as well as a biotin or hapten moiety that binds with high affinity to avidin or an anti-hapten antibody, respectively. Similarly, avidin, streptavidin, NeutrAvidin biotin-binding protein and CaptAvidin biotin-binding protein (Section 7.5) can tightly bind up to four molecules of a biotinylated target, and immunoglobulin G (IgG) can bind up to two haptens.



**Figure 5.2** T-2556 tris-(2-carboxyethyl)phosphine, hydrochloride.



**Figure 5.3** SPDP derivatization reactions. SPDP (S-1531) reacts with an amine-containing biomolecule at pH 7 to 9, yielding a mixed disulfide. The mixed disulfide can then be reacted with a reducing agent such as DTT (D-1532) or TCEP (T-2556) to release a pyridyldithiopropionyl conjugate or with a thiol-containing biomolecule to form a disulfide-linked biomolecule pair. Either reaction can be quantitated by measuring the amount of 2-pyridylthione chromophore released during the reaction.

*We find that the combination of SPDP and SMCC labeling is usually most reliable for forming stable protein–protein chemical crosslinks.*

## 5.2 Chemical Crosslinking Reagents

The most common schemes for forming a heteroconjugate involve the indirect coupling of an amine group on one biomolecule to a thiol group on a second biomolecule, usually by a two- or three-step reaction sequence. The high reactivity of thiols (Chapter 2) and — with the exception of a few proteins such as  $\beta$ -galactosidase — their relative rarity in most biomolecules make thiol groups ideal targets for controlled chemical crosslinking. If neither molecule contains a thiol group, then one or more can be introduced using one of several thiolation methods. The thiol-containing biomolecule is then reacted with an amine-containing biomolecule using a heterobifunctional crosslinking reagent such as one of those described in Amine–Thiol Crosslinking, below.

### Thiolation of Biomolecules

#### Introducing Thiol Groups

Several methods are available for introducing thiols into biomolecules, including the reduction of intrinsic disulfides, as well as the conversion of amine, aldehyde or carboxylic acid groups to thiol groups:

- Disulfide crosslinks of cystines in proteins can be reduced to cysteine residues by dithiothreitol<sup>1</sup> (DTT, D-1532), tris-(2-carboxyethyl)phosphine (TCEP, T-2556; Figure 5.2) or tris-(2-cyanoethyl)phosphine (T-6052). However, reduction may result in loss of protein activity or specificity. Excess DTT must be carefully removed under conditions that prevent reformation of the disulfide,<sup>2</sup> whereas excess TCEP usually does not need to be removed before carrying out the crosslinking reaction. TCEP is also stable at higher pH values than is the air-sensitive DTT reagent.
- Amines can be indirectly thiolated by reaction with succinimidyl 3-(2-pyridylthio)propionate<sup>3</sup> (SPDP, S-1531), followed by reduction of the 3-(2-pyridylthio)propionyl conjugate with DTT or TCEP (Figure 5.3). Reduction releases the 2-pyridylthione chromophore, which can be used to determine the degree of thiolation.
- Amines can be indirectly thiolated by reaction with succinimidyl acetylthioacetate<sup>4</sup> (SATA, S-1553), followed by removal of the acetyl group with 50 mM hydroxylamine or hydrazine at near-neutral pH (Figure 5.1). This reagent is most useful when disulfides are essential for activity, as is the case for some peptide toxins.
- Thiols can be incorporated at carboxylic acid groups by an EDAC-mediated reaction with cystamine, followed by reduction of the disulfide with DTT or TCEP;<sup>5,6</sup> see Amine–Carboxylic Acid and Thiol–Carboxylic Acid Crosslinking below.
- Tryptophan residues in thiol-free proteins can be oxidized to mercaptotryptophan residues, which can then be modified by iodoacetamides or maleimides.<sup>7–9</sup>

Our preferred reagent combination for protein thiolation is SPDP/DTT or SPDP/TCEP.<sup>10</sup> Molecular Probes uses SPDP to prepare a reactive R-phycoerythrin derivative (P-806, Section 6.4), providing researchers with the optimal number of pyridyldisulfide groups for crosslinking the phycobiliprotein to thiolated antibodies, enzymes and other biomolecules through disulfide linkages.<sup>11</sup> More commonly, the pyridyldisulfide groups are first reduced to thiols, which are then reacted with maleimide- or iodoacetamide-derivatized proteins (Figure 5.3, Figure 5.4). SPDP can also be used to thiolate oligonucleotides<sup>12</sup> and — like all of the thiolation reagents in this section — to introduce the