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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.

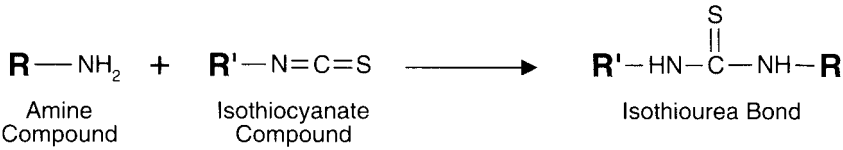
Ultimately, this section is meant to function as a ready-reference database for learning or review of bioconjugate chemistry. In this regard, a reaction can be quickly found, a short discussion of its properties and use read, and a visual representation of the chemistry of bond formation illustrated. 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.

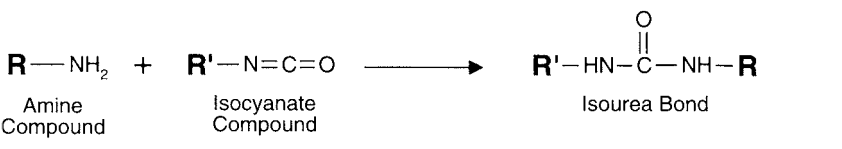


(Reaction 1)

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.

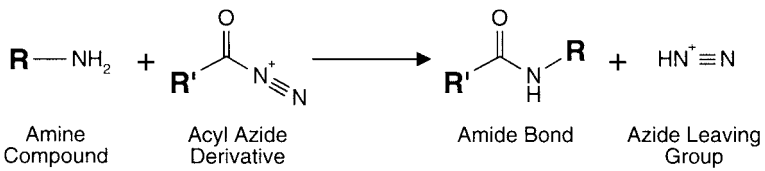


(Reaction 2)

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)

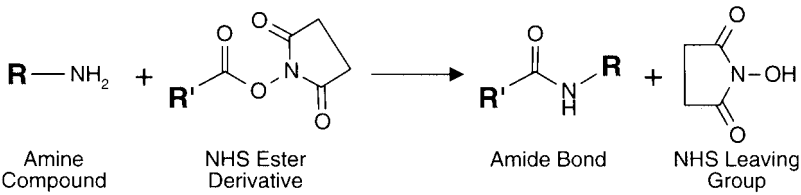
The major competing reaction in acyl azide coupling is hydrolysis. The higher the pH, the faster the reactivity, both with regard to amine conjugation and hydrolysis. Crosslinkers or modification reagents containing this compound must be kept dry to preserve activity. Reactions are complete in 2–4 hours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