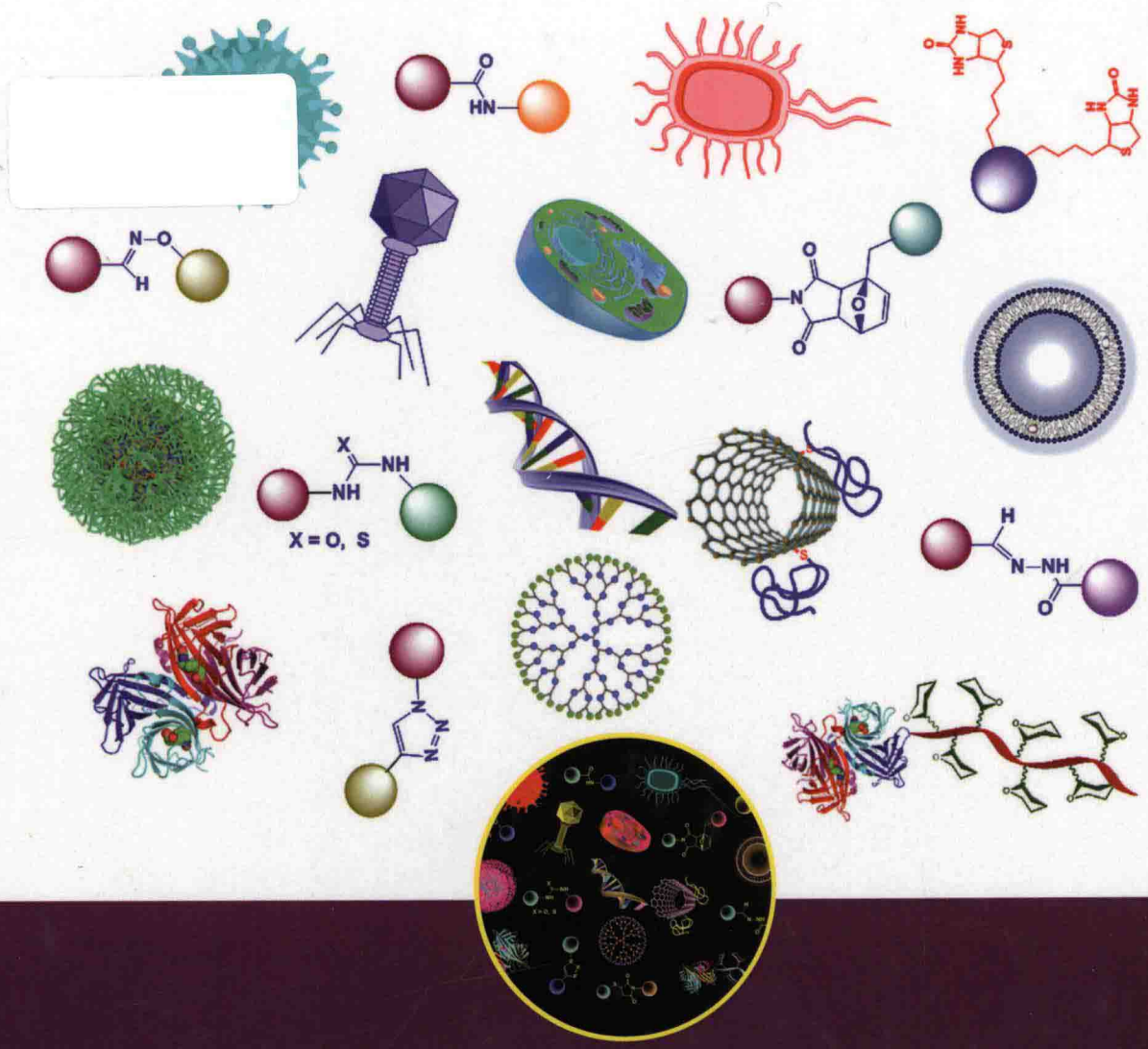

CHEMISTRY OF BIOCONJUGATES

SYNTHESIS, CHARACTERIZATION, AND BIOMEDICAL APPLICATIONS

Edited by

RAVIN NARAIN

WILEY

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Synthesis, Characterization, and Biomedical Applications

Edited by

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CHEMISTRY OF BIOCONJUGATES

PREFACE

Combining characteristics of different components into one to generate new molecular systems with unique properties by simply linking one or more (macro)molecules is defined as bioconjugation. The ability to create such biohybrids either covalently or non-covalently has allowed major breakthrough in many industrial and biomedical areas such as bioseparation, targeting, detection, biosensing, biological assays, etc. This book provides a comprehensive account on the chemistries involved in the formation of bioconjugates, followed by an extensive review of all the different types of bioconjugates generated so far from polymers, dendrimers, nanoparticles, carbon nanotubes, hydrogels and so on for different bio-related applications. A section is also devoted to the physicochemical and biochemical properties of bioconjugates. Finally, the book also provides a comprehensive account on the significance of bioconjugation which is lacking in many of the current available resources.

The book begins by providing an overview of the chemistry involved in bioconjugation. Different types of bioconjugation strategies available for the modification of biomolecules (proteins, peptides, carbohydrates, polymers, DNA) are presented. Classical bioconjugation approaches are described first, followed by some recent bioconjugation techniques. This section also provides detailed synthetic protocols for some of the most important strategies for bioconjugation.

Polymer bioconjugates are then discussed separately in three sections, namely polyethylene glycol (PEG), synthetic polymer bioconjugates, and natural polymer bioconjugates. PEG has been extensively used in the development of macromolecular therapeutics and most of the current clinically available therapeutics are PEGylated bioconjugates. PEGylation has been used for proteins, anticancer drugs, and other bioactive molecules such as peptides, antibodies, oligonucleotides, aptamers, red blood cells, and more recently, viruses. Conjugation of synthetic polymers to biomolecules is an appealing strategy to produce new biomacromolecules with distinctive properties. Typical conjugation strategies are either "*grafting from*" or "*grafting to*" approaches. In "*grafting from*" approach, monomer-functionalized biomolecules are polymerized to produce synthetic polymer bioconjugates. On the other hand, in "*grafting to*" approach, biomolecules are immobilized by reactive coupling reactions. Random and site-specific modifications of natural macromolecules have also been extensively studied and, therefore, an elaborated section has been devoted to this area.

The next section is focused on organic nanoparticle bioconjugates. Different chemical strategies used to couple biomolecules with liposomes, micelles, carbon nanotubes, fullerene, and graphene are discussed. Bioconjugation of biomolecules to those organic nanoparticles has become increasingly important in drug formulation and therapeutic delivery. Choosing the right chemistry between the biomolecule and organic nanoparticle has been the focus of great attention in recent years in view of improving the sustained delivery of these bioconjugates to the targeted site effectively. Carbon nanotubes, fullerene, and graphene have unique properties and their coupling with biomolecules have generated unique materials of high potency in biomedical applications.

Inorganic nanomaterials such as gold, iron oxide, quantum dots, and silica have become key players in the biomedical field. Their unique chemical and physical properties have contributed significantly in further development of these nanomaterials. Their surface properties dictate their colloidal stability and biocompatibility. Therefore, in recent years, several strategies have been developed to conjugate bioactive

molecules, targeting ligands and other biologically relevant molecules to broaden the applications of these nanomaterials. This section discusses the different chemistries used in bioconjugation of biomolecules on the surface of these most widely used inorganic nanomaterials.

With the rapid development of the chemistries in bioconjugation, it is now possible to prepare cell-based bioconjugates efficiently. Modifying cell surfaces with bioactive molecules or synthetic polymers has been a versatile way to add advanced features and unique properties to inert cells. Creating a nanoscale layer on a cell surface, for example, significantly improves or even completely changes its biological properties as well as introduces new unique properties, such as chemical functionality, surface roughness, surface tension, morphology, surface charge, surface reflectivity, surface conductivity, and optical properties. Recently, surface modification of living cells has been the subject of study for a variety of biological applications such as imaging, transfection, and control of cell surface interactions. Additionally, microgels and hydrogels have emerged as important materials due to their unique features such as encapsulation, swelling, degradation, and controlled dimensions. Such features are further enhanced by conjugating them chemically or physically with other bioactive molecules. This section reviews different approaches in making those biologically relevant bioconjugates. Subsequently, various conjugation strategies for the preparation of carbohydrate-based vaccines and different types of chemistry used for covalent linkage of the individual vaccine components are discussed. Then, both direct and indirect conjugation techniques, as well as different types of linker molecules used to generate the spacing deemed required between carbohydrate and immunogen are presented.

Finally, once the bioconjugates are synthesized, their structures and function need to be properly characterized to fully understand their properties. Therefore, proper tools are required to fully understand the properties of these complex hybrid biomolecules. The techniques used in the full characterization of these bioconjugates are discussed in detail. This section also focuses on the physicochemical and biochemical properties of bioconjugates. The physical properties of conjugates, including their response to temperature, external field (magnetic field, electric field, ultrasound), and light, are discussed. The chemical properties of conjugates, such as their response to a change in the pH and ionic strength, are also summarized. Additionally, the properties of conjugates in response to glutathione (GSH), hydrogen peroxide (H_2O_2), and glucose are also outlined. These bioconjugates have been implied for a variety of biological applications, including drug and gene delivery applications, biological assays, imaging, and biosensors. The success of these bioconjugates in research laboratories, compared to their precursor biomolecules, has further encouraged their use for industrial applications. Some of these bioconjugates are now used in clinical trials.

Ravin Narain

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CONTENTS

SECTION I

GENERAL METHODS OF BIOCONJUGATION

1

COVALENT AND NONCOVALENT BIOCONJUGATION STRATEGIES

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1.1 INTRODUCTION

Bioconjugation—the process of covalently or noncovalently linking a biomolecule to other biomolecules or small molecules to create new molecules—is a growing field of research that encompasses a wide range of science between chemistry and molecular biology. The tremendous achievement of modern synthetic organic chemistry has led to a variety of bioconjugation techniques [1] available for application in research laboratories, medical clinics, and industrial facilities. While bioconjugation involves the fusion of two biomolecules, for example protein–protein, polymer–protein, carbohydrate–protein conjugates, it also involves the attachment of synthetic labels (isotope labels, fluorescent dyes, affinity tags, biotin) to biological entities such as carbohydrates, proteins, peptides, synthetic polymers, enzymes, glycans, antibodies, nucleic acids, and oligonucleotides (ONTs). The product of a bioconjugation reaction is usually termed as a "*bioconjugate*" and synthetic macromolecules produced by bioconjugation approaches are commonly referred to as *biohybrids*, *polymer bioconjugates*, or *molecular chimeras*. Modification of biomolecules is an important technique for modulating the function of biomolecules and understanding their roles in complex biological systems [1a]. However, selective biomolecule modification remains challenging and the ease of generating the desired bioconjugate rapidly under physiological conditions is vital for many applications, such as disease diagnosis, biochemical assays, ligand discovery, and molecular sensing. As applications of bioconjugates continue to grow, an expanded toolkit of chemical methods will be required to add new functionality to specific locations with high yield and chemoselectivity.

The aim of this chapter is to provide a comprehensive review of the different types of bioconjugation methods (covalent and noncovalent approaches) available for the modification of biomolecules (proteins, peptides, carbohydrates, polymers, DNA, etc.). Traditional bioconjugation methods will first be elaborated upon, followed by some modern bioconjugation techniques, particularly the emerging role of bioorthogonal chemistry, where the translation of knowledge of chemical reactions to reactions in living systems can be achieved. While the synthetic aspects of the bioconjugates will be the main focus, a brief description of their applications will also be presented.

1.2 COVALENT BIOCONJUGATION STRATEGIES

The covalent bond is the most common form of linkage between atoms in organic chemistry and biochemistry. The reaction of one functional group with another leads to the formation of a covalent bond via the sharing of electrons between atoms (Figure 1.1).

Covalent bioconjugation strategies are generally categorized as random (modification at multiple sites) or site-specific (modification at a single site) bioconjugation. Traditional covalent bioconjugation strategies preclude control over the regiochemistry of reactions, thereby leading to heterogeneous reaction products and eventually, loss of the

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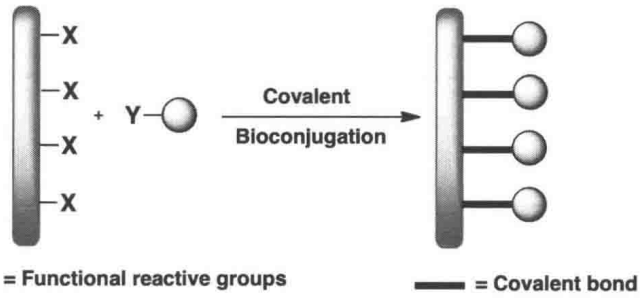


FIGURE 1.1 Schematic representation of covalent bioconjugation strategy.

biological function of the target biomolecule [1(d)]. However, new methods of bioconjugation that are highly site specific and cause minimal change to the active form of the biomolecule have been developed. For instance, *bioorthogonal* reactions have recently emerged as essential tools for chemical biologists [1(e)]. The following sections survey the covalent modifications of several reactive functional groups (carboxylic acids, aldehydes, ketones, amines, thiols, and alcohols), which are generally present or can be introduced onto macromolecules (proteins, peptides, carbohydrates, nucleic acids, ONTs, etc.).

1.2.1 Carboxyl Modifications

Carboxyl groups are commonly found on the C-terminal ends of proteins and on glutamate (Glu) and aspartate (Asp) amino acid side chains. Carboxylic acids are strong organic acids and the fastest reaction with a nucleophile is removal of the acidic hydrogen to form the carboxylate anion. The resulting anion is resistant to addition reaction with a second nucleophile, and thus makes conjugation through carboxylate group via nucleophilic addition a difficult process. Usually, harsher conditions, acid catalysis, or special reagents are required to promote carboxylic acid-mediated reactions. However, some carboxylate-reactive chemical reactions have been achieved with diazoalkanes and diazoacetyl derivatives (diazoacetate esters and diazoacetamides) and common activating agents such as carbonyldiimidazole (CDI) and carbodiimides to derivatize carboxylic acids. These reactions generate stable covalent linkages namely esters and amides.

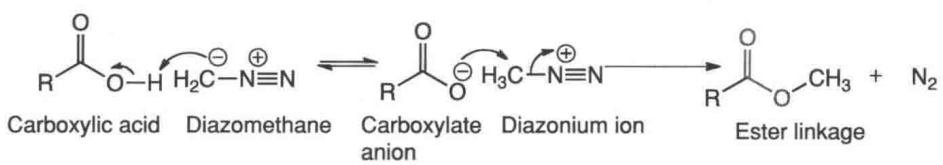
1.2.1.1 Diazoalkanes and Derivatives Diazoalkanes, in particular, diazomethane [2] is a powerful reagent for esterification of carboxylic acids. They react instantaneously with carboxylic acids without the addition of catalysts and may be useful for direct carboxylic acid modification of proteins and synthetic polymers. The reaction mechanism involves nucleophilic attack of the resulting carboxylate anion onto the diazonium ion, followed by an alkylation step to furnish a covalent ester linkage. The driving force of the reaction is the formation of nitrogen, which is a superb leaving group (Scheme 1.1).

Diazomethane, though easily made, is quite toxic, highly explosive, and requires special glassware for reactions. A less explosive and commercially available reagent, trimethylsilyldiazomethane [3], is commonly employed; however, toxicity is still a major concern. In the past, fluorescent diazomethane derivatives have gained much attention for the derivatization of biologically important molecules, especially the nonchromophoric fatty acids [4], bile acids, and prostaglandins. 9-anthryldiazomethane (ADAM) [5, 6] and 1-pyrenyldiazomethane (PDAM) [7, 8] are diazomethane derivatives of the fluorescent dyes anthracene and pyrene, respectively, that have commonly been used as fluorescent labeling reagents for liquid chromatographic determination of carboxylic acids. ADAM and PDAM react readily with carboxylic acids at room temperature in both protic and aprotic solvents. ADAM was found to be unstable and decomposed easily upon storage, while PDAM has a much better chemical stability (a 0.1% (w/v) of PDAM in ethyl acetate solution is stable for 1 week at $\leq -20°C$) [9]. Furthermore, the detection limit for PDAM conjugates (about 20–30 fmol) is reported to be five times better than reported for detection of ADAM conjugates. Fatty acids derivatized with these reagents have been used to measure phospholipase A_2 activity [10].

Protocol for reaction of PDAM with fatty acids [9]:

1. Add 100 μL of 1 mg/mL solution of PDAM in ethyl acetate (ethyl acetate stock solution) to 100 μL of 0.01–10 μg/mL fatty acid solution in methanol.
2. React for 90 minutes at room temperature.
3. Inject 5 μL of reaction mixture into an HPLC column.

1.2.1.2 Activating Agents The direct conversion of a carboxylic acid to an amide with amines is a very difficult process as an acid–base reaction to form a carboxylate ammonium salt occurs first before any nucleophilic substitution reaction happens. As such, amide formation from carboxylic acid is much easier if the acid is first activated (Scheme 1.2)



SCHEME 1.1 Mechanism of diazomethane esterification reaction.

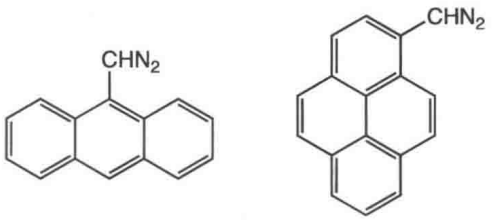


FIGURE 1.2 Fluorescent diazomethane derivatives as labeling reagents.

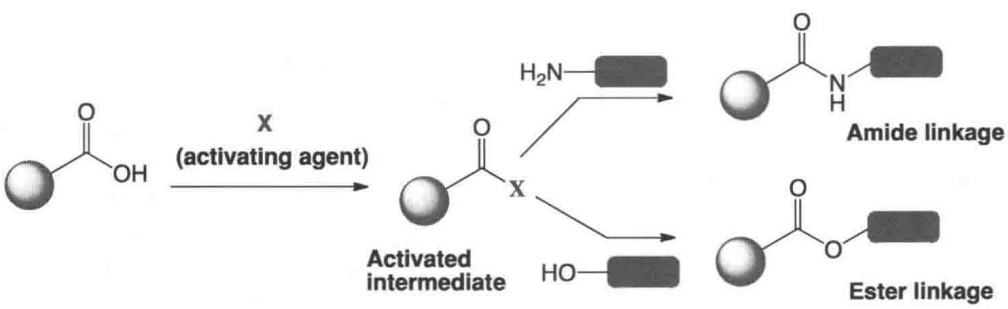
prior to nucleophilic attack by the amine. This strategy converts the poor carboxy —OH leaving group into a better one. Ester linkages can also be formed using this strategy in the presence of alcohols.

The explosion in the field of peptide chemistry has led to the development of many activating agents that greatly enhance amide formation, but only the most commonly used ones, such as CDI and carbodiimides, will be discussed here (Table 1.1). *N, N′*-Carbonyldiimidazole (CDI) [11] is a white crystalline solid that is useful for activating carboxylic acids to form amide, ester, and thioester linkages. During the reaction, a reactive intermediate, *N*-acylimidazole is formed with liberation of carbon dioxide and imidazole as innocuous side products. The *N*-acylimidazole can then react with amines or alcohols to form stable covalent amide or ester linkages, respectively. CDI is not commonly used in routine peptide synthesis, but nevertheless is quite useful for coupling peptide fragments to form large peptides and small proteins [12]. One unique application of CDI is the synthesis of urea dipeptides [13]. Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) are commonly used in organic synthesis for the preparation amides, esters, and acid anhydrides from carboxylic acids. These reagents can also transform primary amides to nitriles, which is a somewhat troublesome side reaction of asparagine and glutamine residues in peptide synthesis. The choice of these carbodiimides depends largely on their solubility properties. DCC was one of the first carbodiimides developed [14] and is widely used in peptide synthesis. It is highly soluble in dichloromethane, acetonitrile, dimethylformamide (DMF), and tetrahydrofuran, but is insoluble in water. The by-product of a DCC-mediated reaction is dicyclohexylurea, which is nearly insoluble in most organic solvents and precipitates from the reaction mixture as the reaction progresses. Thus, DCC is very useful in solution-phase reactions, but is not appropriate for reactions on resin. Another drawback of DCC-mediated coupling is that trace amounts of dicyclohexylurea remains and are often tedious to remove. DIC was developed as an alternative of DCC since being a liquid, it is easier to handle and also forms a soluble urea by-product, which can easily be removed by simple extraction [15].

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC or EDAC) is a versatile modern coupling reagent. It is commonly known as a zero-length cross-linking agent used to conjugate carboxyl groups and amines to form stable covalent amide linkages. Amide bonds typically have a half-life of circa 600 years in neutral solution at room temperature [16], and this extraordinary stability renders amide linkages to be very attractive for bioconjugation. This carbodiimide reagent and its urea by-product are both water soluble; hence, the by-product and any excess reagent are removed by aqueous extraction. EDC reacts with a carboxyl to form an amine-reactive *O*-acylisourea intermediate, which is highly unstable and short-lived in aqueous solution. Thus, hydrolysis is a major competing reaction. It was found that the addition of *N*-hydroxysulfosuccinimide (Sulfo-NHS) stabilizes the amine-reactive intermediate by converting it to a semistable amine-reactive Sulfo-NHS ester (Scheme 1.3), thereby increasing the efficiency of EDC-mediated coupling reactions [17].

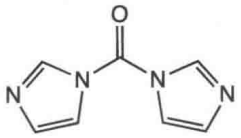
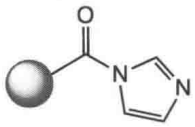
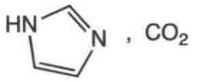
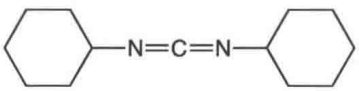
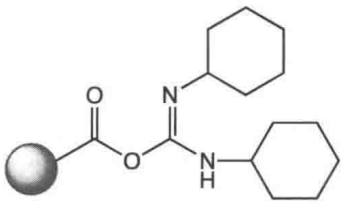
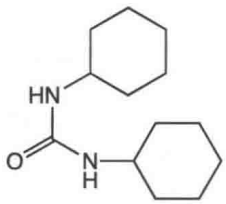
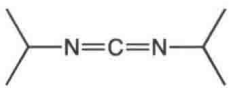
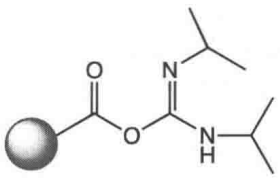
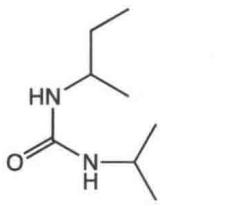
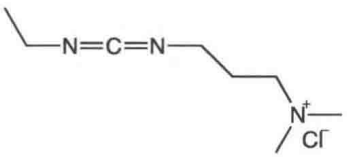
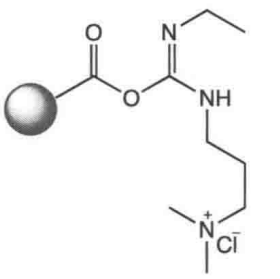
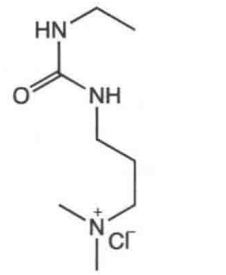
Protocol for conjugation of proteins with EDC and Sulfo-NHS [18]:

1. Add EDC (~2 mM) and Sulfo-NHS (~5 mM) to protein #1 solution.
2. React for ~15 minutes at room temperature.
3. Add 2-mercaptoethanol (final concentration of 20 mM) to quench the EDC.
4. Optional step: Separate the protein from excess reducing agent and inactivated cross-linker using a Zeba



SCHEME 1.2 General strategies for the conjugation of carboxylic acid with amines or alcohols via an activating agent.

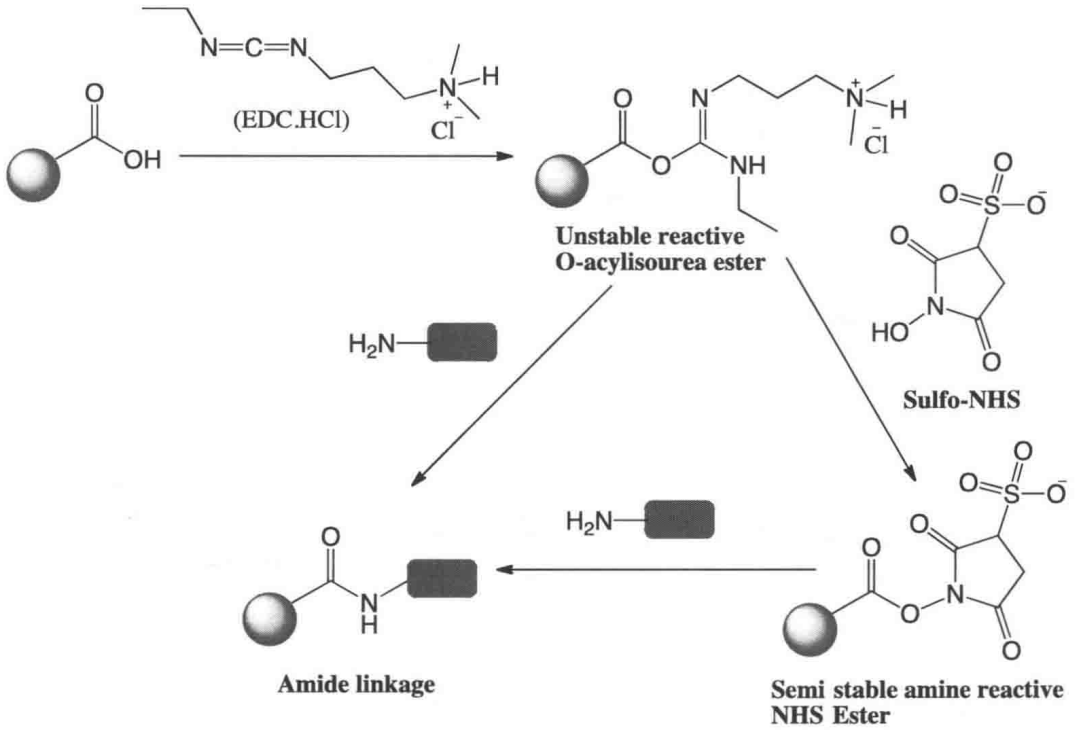
TABLE 1.1 Common Activating Agents for Carboxyl-reactive Groups

Activating Agents	Active Intermediates	By-products
		
N, N'-Carbonyldiimidazole (CDI)	N-Acylimidazole	Imidazole
		
Dicyclohexylcarbodiimide (DCC)	O-Acylisourea	Dicyclohexylurea
		
Diisopropylcarbodiimide (DIC)	O-Acylisourea ester	Diisopropylurea
		
1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC)	O-Acylisourea ester	Urea derivative

Desalting Spin Column. Equilibrate the column with activation buffer.

5. Add protein #2 to the reaction mixture or the pooled fractions containing the activated protein at an amount equal to the number of moles of protein #1.
6. React for 2 hours at room temperature.
7. Add hydroxylamine to a final concentration of 10 mM to quench the reaction. (Other means of quenching involve adding 20–50 mM Tris, lysine, glycine, or ethanolamine; however, these primary amine-containing compounds will result in modified carboxyls on protein #1).
8. Remove excess quenching reagent by gel filtration using the same type of column as in Step 4.

A major drawback of carbodiimide activation of amino acid derivatives is that it usually leads to partial racemization of the amino acid. In peptide synthesis, an equivalent of an additive such as triazoles (e.g., 1-hydroxy-7-aza-benzotriazole [19]) is added to minimize this racemization problem. Recently, during the development of prodrugs for the antitumoral agent thiocoraline, a new coupling reagent known as *N,N,N',N'*-tetramethylchloroformamidinium hexafluorophosphate (TCFH) [20] was developed for the coupling of the carboxylic group of an amino acid with the quinolic alcohol to generate an ester linkage (Scheme 1.4). In this case, standard coupling reagents and procedures failed to afford the desired target derivatives. A number of conjugates including PEGylated derivatives with higher solubility were synthesized using the TCFH method.



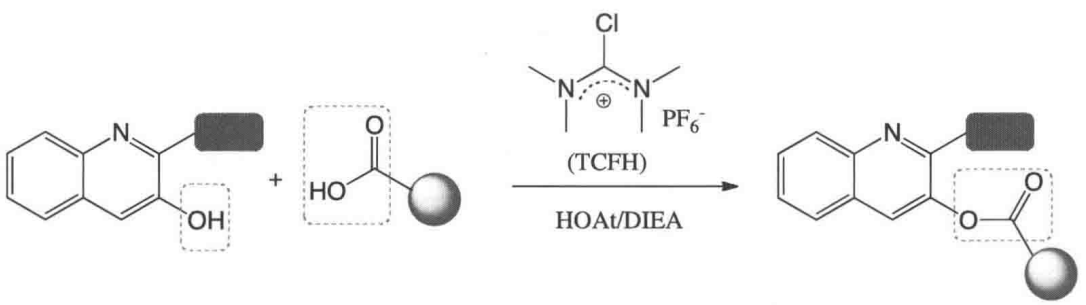
SCHEME 1.3 EDC-mediated protein–carboxylic acid conjugation.

1.2.2 Carbonyl Functional Groups

Aldehydes and ketones are organic compounds that incorporate a carbonyl group (C=O) and are good electrophiles. As such, they undergo nucleophilic addition reactions with various nucleophiles such as amines, *N*-alkoxyamines (or aminooxy groups), hydrazines, or hydrazide to generate products linked by imine-, oxime-, and hydrazone-reactive groups respectively (Scheme 1.5). The facile synthesis of these carbon–nitrogen double bonds in aqueous solutions at neutral pH makes them attractive for bioconjugation and thus, they have found widespread applications in chemical biology, mainly for the synthesis of nucleic acid conjugates [21]. Aldehydes and ketones are also known as chemical reporters that can tag proteins [22], glycans [23], and other secondary metabolites.

1.2.2.1 Conjugation via Reductive Amination Reductive amination [24] is a process that transforms a carbonyl group (typically aldehydes and ketones) into an amine via an intermediate imine (Schiff base). Under acidic conditions, the carbonyl group first reacts with primary amines to form a hemiaminal species, which subsequently loses a water molecule to generate a reversible unstable imine. The imine is then trapped irreversibly with a reducing agent in a one-pot reaction to afford a stable amine product (Scheme 1.6). The overall two-step sequence is called reductive amination. Borohydrides are common reducing agents with sodium cyanoborohydride ($NaBH_3CN$) being the most widely used due to its high selectivity to imines and relative unreactivity with oxo groups [25]. Sodium triacetoxyborohydride, $NaBH(OAc)_3$, was introduced as an alternative mild and nontoxic reducing agent of $NaBH_3CN$ [26].

The reaction of a carbonyl group with an amine proceeds with high chemoselectivity and is also compatible with many functional groups present in biomolecules. Carbohydrate–protein conjugates play vital roles in both



SCHEME 1.4 TCFH-mediated conjugations for ester linkage.