Chemoenzymatic Bioconjugation of Antibodies: Linking Proteins for Biomedical Applications

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Abstract

Antibodies are useful biomolecules applied in many biomedical applications. The selectivity and specificity of antibodies against the target antigens have gained wide interest for both diagnostic and therapeutic applications. The antibodies are capable of functioning as target-specific carriers to allow site-specific delivery of payloads. However, the challenge has always revolved around the ability to attach designer proteins, enzymes, or drugs to the antibody molecule. The conventional approach involves the use of chemical-based modifications with the introduction of chemical linkers and alteration of chemical functional groups to initiate a covalent attachment of molecules to the antibodies. However, the use of chemically modified strategies to attach antibodies to various molecules has provided several setbacks throughout the years. The major consideration involves the conjugation efficiency, the yield of conjugated product recovered post-conjugation, and more importantly the effects to the antibody-binding sites. Therefore, the introduction of bioconjugation approaches utilizing biologically active enzymes to initiate conjugation processes provided researchers with a much-anticipated alternative that was less toxic to the native proteins. This chapter focuses on the application of biologically inspired enzymes that have been used successfully to conjugate proteins or drugs to antibodies in a "green" manner. The enzymes highlighted in this chapter would include sortase,

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transglutaminase, and formylglycine-generating enzymes. The chapter also highlights the applications of these methods to generate conjugates that have been applied either for diagnostic or therapeutic application.

Keywords

Antibody · Chemoenzymatic · Formylglycine-generating enzyme · Sortase · Transglutaminase

18.1   Introduction

Emil von Behring and Shibasaburo Kitasato mentioned "blood is an unusual fluid" in the 1980s when they first discovered substances in serum that are able to neutralize tetanus and diphtheria. Later, they found that injection of the antitoxin serum into infected animals helped the animals to neutralize those infections (von Behring and Kitasato 1890). This is the first reference of antibodies where serum therapy was applied in medical treatment. However, the term "antibody" was first coined by Paul Ehrlich in year 1891, who proposed the production of factors in human serum upon exposure to foreign materials to neutralize these foreign substances in his side-chain theory (Dale 2012; Johnston et al. 2016). A great leap in progress was achieved in the development of monoclonal antibody technology when Kohler and Milstein introduced hybridoma technology in 1975. The fusion of myeloma cells with B cells from spleen cells of immunized mouse is able to generate immortal hybridoma cells, which are able to produce monoclonal antibodies (mAbs) against a specific antigen continuously (Köhler and Milstein 1975). Therefore, monoclonal antibodies have become one of the most important classes of therapeutic molecules covering most of the therapeutics' market over the past three decades. The advancement of recombinant DNA technology has allowed the isolation of high-affinity binders against antigens, and the antibody engineering allowed the refinement of antibodies for better pharmacokinetics (Frenzel et al. 2016). Both conjugated and non-conjugated antibodies have been approved by the US Food and Drug Administration (FDA) to be applied for the treatment of various diseases (Ornes 2013; Perez et al. 2014). However, unconjugated antibodies have been reported to be therapeutically less effective and also less potent than conjugated antibodies (Panowski et al. 2014; Sharkey and Goldenberg 2008). Cytotoxic drug MMAE-conjugated antibodies (SGN-35) and anti-CD19-idarubicin are examples of antibody-drug conjugates (ADCs), which demonstrated a better activity compared to its unmodified form (Rowland et al. 1993; Younes et al. 2010). Reports showed numerous molecules have been conjugated to antibodies, such as drug (Strop et al. 2016), antibiotic (Mariathasan and Tan 2017), radioisotope (McCracken and Radu 2015), polyethylene glycol (Wen et al. 2001), biotin (Josten et al. 2000), toxin (Kornberger and Skerra 2014), enzyme (Ismail and Lim 2016), peptide (Tong et al. 2013), DNA (Gong et al. 2016), and nanoparticles (Jazayeri et al. 2016). The

wide range of conjugated molecules highlights the robustness of antibodies to withstand modifications making it ideal for diagnostics and therapeutics.

The antibody-drug conjugate is one of the most important classes of antibody conjugates that harness the combined advantages of both antibody specificity and potency of conjugated molecules. Generally, ADC is made up of three main components: antibody, linker, and payloads. This combination could potentially reduce the toxicities induced by the bystander effect while enhancing the therapeutic efficacy (McCombs and Owen 2015). For example, cytotoxic drug MMAE-conjugated antibodies (SGN-35) demonstrated better activity compared to its unconjugated form against CD30 by providing an additional effector mechanism (Younes et al. 2010). Another example was shown by anti-CD19-idarubicin conjugate, which performed a higher antileukemia efficacy with a lower toxicity when compared to the unconjugated idarubicin at similar dosages (Rowland et al. 1993). Apart from enhancing the therapeutic efficacy of antibodies, antibody conjugation can also prolong the circulating half-lives and reduce the immunogenicity. This could be achieved via either random or site-specific covalent conjugation of linear or branched water-soluble polymer, such as polyethylene glycol (PEG), to antibodies (Bailon and Won 2009; Chapman 2002; Pasut and Veronese 2012). Limitations of naked antibodies in therapeutics often lead to non-curative therapies, especially in cancer treatments. The inability and challenges of naked antibodies to achieve targeted therapeutic efficacy have urged researchers to venture into antibody conjugation. Various molecules were therefore made available to be conjugated to the antibodies depending on the final use of the antibodies. Up till today, only four commercial antibody conjugates (i.e., Mylotarg® Withdrawal, Adcetris®, Kadcyla®, and Zevalin®) have successfully made their way to market, and many more are still under clinical trials (Dennler et al. 2015; Panowski et al. 2014). Gemtuzumab ozogamicin (GO, trade name: Mylotarg®) was the first ADC to obtain regulatory approval to be marketed in the USA in the year 2000. GO is a humanized anti-CD33 antibody conjugated with the calicheamicin derivative, which could be used for the treatment of acute myeloid leukemia (AML) (Hamann et al. 2002). Upon intracellular release, GO induces cell death by breaking up DNA strands and causing apoptosis (Cowan et al. 2013). However, GO was voluntarily withdrawn from the market 10 years later by Pfizer due to the concerns that it lacks clinical benefits to patients (Pharma 2010). Generally, antibody conjugation can be performed via either chemical modifications or bioconjugation by targeting a few sites on antibodies, such as specific amino acid residues, carbohydrate moiety, N-terminal of heavy and light chains, Fc-binding domains (FcBD), and nucleotide-binding site of antibodies (Dennler et al. 2015).

18.2 Conventional Chemical Conjugation Methods

Chemical conjugation is a conventional method which is commonly used to produce antibody conjugates. Various chemical reagents have been applied in modifying sites on antibodies to become reactive. The sites that are generally targeted in

antibodies could be categorized into four main groups: amines, thiols, sugar alcohols, and carboxylic acids (Sesay 2003). Among these sites, amines and thiols are widely applied and discussed in most of the publications due to the attractiveness of surface-exposed lysine residues (amine groups) and interchanged cysteine residues (thiol groups) in conjugation. Adcetris® and Kadcyla® are the examples of FDA-approved ADC products, which are conjugated via lysine and cysteine residues, respectively (Sochaj et al. 2015).

18.2.1 Lysine Conjugation

Lysine conjugation is one of the most widely used non-specific conjugation strategies in antibodies due to its abundance. About 80–100 lysine residues are present in a standard immunoglobulin (IgG). Besides that, lysine residues are exposed on the surfaces of antibodies, making them accessible for reaction. Apart from that, aliphatic ε-amine group of lysine is a good source of nucleophiles that makes lysine reacts easily with reagents to form stable bonds (Brun and Gauzy-Lazo 2013; Sesay 2003). Lysine conjugation could be achieved via either one-step or two-step conjugation. One-step conjugation directly generates amide bonds between lysine ε-amine groups of the antibody with the amine-reactive group found on the desired molecules, while two-step conjugation starts with the modification of lysine residues on antibodies using bifunctional reagent prior to amide bond formation with the reactive groups of the desired molecules (Brun and Gauzy-Lazo 2013). Kadcyla® is an example of FDA-approved antibody conjugate for breast cancer, which is generated by two-step lysine conjugation, where linker and payload are conjugated separately (Jackson 2016). Several strategies have been commonly applied to achieve lysine conjugation, which are summarized as below. N-hydroxyl-succinimidyl (NHS) ester is the most common reagent used in one-step lysine conjugation method to form amide bond between the carboxylic acid and amino groups. The simplicity and availability of NHS have made it gained popularity for application in lysine-based conjugation. In this reaction, NHS esters form irreversible amide bonds with the primary amine of lysine to release NHS under alkaline conditions (i.e., pH 7.2–9). Another one-step lysine conjugation method is the isothiocyanate method, in which isothiocyanates react with primary amines to yield thiourea and urea derivatives. Fluorescein isothiocyanate (FITC) is a well-known example of isothiocyanate derivative that is widely used for antibody labeling. Compared to NHS esters, isothiocyanates are more stable because it is less prone to decomposition during storage and it ensures reactivity with antibodies after a period of time. However, the reaction condition of isothiocyanate conjugation is more alkaline (about 9–9.5) than NHS ester conjugation, which is unsuitable for alkaline-sensitive proteins (Basle et al. 2010). Hence, Traut's reagent, also known as 2-iminothiolane, provides an alternative in two-step lysine modification via the formation of amide bond. It reacts with the primary amine at alkaline condition (pH 10) to introduce a sulfhydryl (-SH) group while maintaining the charge properties of lysine. The lysine modification via this reagent has the advantage of performing the conjugation without reducing the antibody.

18.2.2 Cysteine Conjugation

Cysteine (Cys) is one of the least abundant amino acid residues, but yet it is found to participate in majority of the protein functional properties (Kim et al. 2015). Due to the low abundance (less than 1.6%), high accessibility (pKa 8–9), and high nucleophilicity of the sulfhydryl (-SH) side chains, cysteine residue serves as one of the most ideal amino acids for bioconjugation (Brotzel and Mayr 2007; Cal et al. 2014). Cysteine conjugation is therefore preferred over lysine because multiple lysine residues are found in an antibody and they are difficult to be controlled for conjugation (Koniev and Wagner 2015). In addition, cysteine conjugation does not have charge issues due to the presence of thiol side chains, and the generated thioether or disulfide bond is generally without charge (Coquerel et al. 2010). Human IgG generally has four interchain solvent-exposed disulfide bonds. Hence, reduction of one disulfide bond gives rise to two thiol groups for conjugation. Up to eight molecules are able to conjugate to an antibody depending on the extent of disulfide reduction. With this method, molecules are therefore attaching to an antibody in even numbers (2, 4, 6, or 8) (Behrens and Liu 2014). Reduction of cysteine disulfide bonds is performed to break the existing disulfide bonds and prevent their reformation. This procedure allows tagging of cysteine residues with different compounds for downstream applications, or introduces reporter groups, such as fluorescent labels (Crankshaw and Grant 2001). Partial reduction with dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) attaches payloads to heavy-light chain disulfides, while 5,5′-dithiobis (2-nitrobenzoic acid) (DNTB) performs partial re-oxidation of fully reduced antibodies to attach ligands to the heavy-heavy chain disulfides (Sun et al. 2005). It has been reported that interchain disulfide bonds are easier to be reduced compared to intrachain disulfide bonds (Schroeder et al. 1981). In some cases, cysteine residues were mutated to other amino acids, such as serine, to produce homogeneous antibody conjugates with defined conjugation sites (McDonagh et al. 2006). The rarity of cysteine residues for conjugation is often explored by the disulfide bond reduction or by the introduction of unnatural cysteine residues via protein engineering. Previous work has demonstrated that reduction of disulfide bonds destabilizes the antibody molecule despite showing no significant structural changes. However, the bioactivity of the antibody was found decreasing or lost due to incomplete disulfide bond formation (McAuley et al. 2008). Several alternative approaches were later proposed to overcome the setbacks of conventional cysteine conjugation by disulfide bridging using dibromomaleimides (Hull et al. 2014; Jones et al. 2012) and bis-sulfone reagents (Badescu et al. 2014). The disulfide bridging increases the stability of antibodies and is reduced from eight to four conjugated molecules (Schumacher et al. 2016). In general, cysteine conjugation is a preferred method compared to lysine due to lower multiplicity compared to lysine residue, which makes the site conjugation easier to be controlled (Koniev and Wagner 2015).

Conjugation of antibodies at cysteine residues could occur via native cysteine residues, engineered cysteine residues, or the introduction of selenocysteine (cysteine analogue). Few strategies have been developed to modify native cysteine

residues using various alkylating reagents, such as α-halocarbonyls, Michael acceptors, and aminoethylation (Chalker et al. 2009). Brentuximab vedotin (anti-CD30) is an example of an antibody conjugate developed via modification of the native cysteine side-chain thiols (Senter and Sievers 2012; van de Donk and Dhimolea 2012). Another alternative is via the introduction of free cysteine residue into antibodies through site-directed mutagenesis. The pros to this approach is that no specialized expression system is required; however, a main consideration with regard to this approach is that cysteine residues cannot be introduced randomly as it will affect the folding or the function of the antibody. Also, the newly introduced cysteine residue can oxidize to form unwanted disulfide bonds with thiol groups that present in the cell (Kline et al. 2015). The incorporation of selenocysteine in antibodies for conjugation is another alternative approach. Selenocysteine is best known as the 21st amino acid, and it is present in all kingdoms of life, as a component of selenoprotein (Johansson et al. 2005). Compared to cysteine, selenocysteine is more reactive toward electrophiles, such as iodoacetamide or maleimide. Hence, selenocysteine is able to couple with electrophile containing agents of antibodies for conjugation (Hofer et al. 2009; Li et al. 2014).

18.2.3 Challenges Associated with Conventional Chemical-Based Methods

Chemical conjugation of antibodies has shown promising results in both diagnostic and therapeutic applications. Despite its practicality, many limitations regarding these conjugation techniques have been reported. Chemical-based conjugation methods often result in batch-to-batch variation due to the heterogeneity in payload-to-antibody ratio (PAR) and poor region selectivity. This is due to the presence of multiple lysine or cysteine residues in antibodies, resulting in random placement of payloads, and the subsequent consequence of random placement allows the generation of heterogeneous antibody conjugates (Dennler et al. 2015). Also, an abundance of amino acid residues, which are available for conjugation, will lead to incomplete reactions and result in a mixture of modified proteins (Coquerel et al. 2010). When heterogeneous antibody conjugates are produced, the unconjugated antibodies compete with the conjugated antibodies for antigen binding, which weakens the therapeutic index of the antibody conjugates (Junutula et al. 2008). In addition, chemical conjugation alters hydrophobicity, polarity, charge, and thermostability of antibodies (Acchione et al. 2012; Boylan et al. 2013; Wakankar et al. 2010). Alteration in charge properties of antibody has been reported to increase the risk of aggregation upon conjugation (Coquerel et al. 2010; Li et al. 2016). Most of the chemical conjugations are performed in an alkaline condition. Therefore, it should be noted that alkaline-sensitive proteins might not be suitable for chemical-based conjugation due to the relatively alkaline conjugation condition (Basle et al. 2010). Also, the confirmation of modification using mass spectrometry (MS) is often required due to the unspecific nature of certain reagents used in chemical

conjugation (Stephanopoulos and Francis 2011). The other setback encountered using chemical conjugation is the low specificity in conjugation due to the reaction with random amino acids. NHS esters are reported to form weak interaction with random amino acids, which are labile to hydrolysis that might produce undesired side effects, if the conjugated drug is released prematurely (Chih et al. 2011). In some cases, site-directed mutagenesis of desired amino acids might affect antigen binding even though the cross-linking of payloads is easier to be managed. This is due to the possible different functional effects as a result of the conjugation to different functional groups (Torres and Casadevall 2008). Another drawback of chemical conjugation is the cost. Selective periodate oxidation of fucose residue was applied to yield aldehyde functional groups to react with the hydrazine-functionalized dolastatin analogue in order to generate homogeneous ADCs. However, this approach was reported to use 100-fold molar excess of the toxin, which is expensive, and it generates hazardous waste during the production (Zuberbühler et al. 2012). To reduce the excessive toxin, another group of researchers had introduced unnatural sugars in the presence of 6-thiofucose when producing their antibodies. However, this approach resulted in heterogeneous ADCs due to the incomplete incorporation of 6-thiofucose (Okeley et al. 2013). Although these approaches are capable of producing ADCs, the quest to generate ADCs at a more cost-effective manner with higher efficacy has led to the introduction of new alternative methods.

18.3 Bioconjugation Using Biological Enzymes

Generally, chemoenzymatic bioconjugation can be divided into three categories: (i) conjugation of protein of interest with an enzyme that recognizes a specific substrate, (ii) labeling the protein of interest with enzyme recognizable motif, and (iii) remodeling the glycan on the protein of interest to introduce payload (McFarland and Rabuka 2015). In this chapter, chemoenzymatic bioconjugation (e.g., sortase, transglutaminase, formylglycine-generating enzyme) of antibodies via labeling the protein of interest with enzyme recognizable motif will be highlighted.

18.3.1 Sortase

Sortase is a transpeptidase, which is produced by Gram-positive bacteria, catalyzing the conjugation of cell surface proteins to bacteria surfaces. Conjugation of virulence factors to bacterial surfaces by sortase and their wide distribution among bacterial pathogens makes it an ideal alternative to develop novel ADCs (Cascioferro et al. 2014; Comfort and Clubb 2004; Garandeau et al. 2002; Pallen et al. 2001). There are four sortase isoforms (i.e., A, B, C, and D) discovered based on the sequence homology and cleavage sites (Comfort and Clubb 2004; Dramsi et al. 2005). Sortase
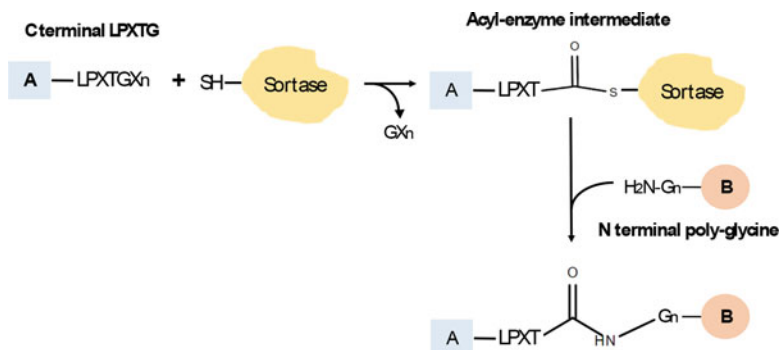
Fig. 18.1 Sortase-mediated protein conjugation between C-terminal LPXTG-tagged protein A and N-terminal poly-glycine-tagged protein B. Sortase forms an acyl-enzyme intermediate with its cysteine residue (SH) by cleaving between threonine and glycine in LPXTG motif. Glycine residues in poly-glycine resolve the intermediate by regenerating the cysteine residue in sortase subsequently conjugating the N-terminal protein

A (SrtA, EC 3.4.22.70) from *Staphylococcus aureus* is particularly well studied among this class of enzymes, which catalyzes the conjugation of proteins containing a C-terminal conserved motif LPXTG and N-terminal penta-glycine motive (Mazmanian et al. 1999; Parthasarathy et al. 2007). As demonstrated in Fig. 18.1, nucleophilic attack of Cys^{184} in SrtA cleaves the LPXTG motif between threonine and glycine to generate an acyl-enzyme intermediate (Navarre and Schneewind 1994; Perry et al. 2002). A second wave of nucleophilic attack on another glycine motive results in the formation of the amide bond with the intermediate molecule to release the SrtA in an unmodified form for the next cycle (Schumacher et al. 2016). Srt-mediated transpeptidation usually performs in a head-to-tail fashion (i.e., N- to C-terminal format). Fusion of the same terminal (N-to-N or C-to-C) is impossible unless the chemical modification was performed on the motifs (Witte et al. 2012; Witte et al. 2013). Sortase bioconjugation platform was recently being incorporated into a commercial conjugation platform (SMAC technology) to generate homogeneous ADCs with predefined DARs of 3.0–3.53. These antibodies showed comparable potency as chemically modified counterparts (Beerli et al. 2015).

18.3.2 Transglutaminase

Transglutaminase (EC 2.3.2.13) is a group of transferases that catalyzes the transfer of acyl groups between γ-carboxyamide groups in glutamine (Glu) residues and ε-amino groups of lysine (Lys) residues, as shown in Fig. 18.2. The reaction forms an isopeptide bond between the two residues, which is relatively resistant to proteolysis degradation (Griffin et al. 2002). Transglutaminase (TGase) is widely found in humans (Suedhoff et al. 1990), animals (Folk and Cole 1966), plants (Del Duca et al. 2014), and microorganisms (Strop 2014). There are eight isoforms of TGase
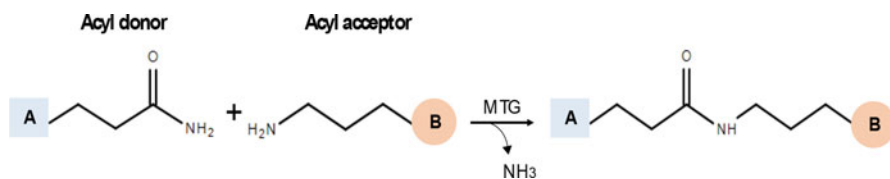
Fig. 18.2 MTG-catalyzed protein cross-linking via transamidation reaction by releasing ammonia in the process

discovered in mammals and all of them require calcium (Ca^+) ions in reactions. The first calcium-dependent TGase discovered in human and animals is transglutaminase 2 (TG2) which is involved in endocytosis, apoptosis, cellular adhesive, and assembly process (Autuori et al. 1998; Chen and Mehta 1999; Shinya et al. 2000; Lorand and Graham 2003). The ability of TGase to cross-link proteins has been exploited in various industries, especially food industries to improve their functional properties (Ikura et al. 1992; Motoki and Nio 1983). Recently, TGase has also been exploited in the pharmaceutical, wool, and leather processing industries. This enzyme was reported to enhance tensile strength and to reduce shrinkage of wool (Tesfaw and Assefa 2014). However, extremely high manufacturing costs, source scarcity, and complexity in downstream applications have prompted the search of a better alternative for mammalian TGase (Kieliszek and Misiewicz 2014). In addition, TGase derived from animals causes red pigmentation, which is detrimental to the product appearance (Yokoyama et al. 2004). When microbial transglutaminase (mTGase) was first isolated in 1989 from *Streptoverticillium* sp., its attributes have gained massive interest among researchers (Ando et al. 1989). This mTGase does not require calcium ions in enzymatic activity and it has a higher thermal stability. Apart from that, it has a lower molecular weight, is stable in a wide range of pH, and contributes to a lower manufacturing cost (Kieliszek and Misiewicz 2014). Therefore, applications of mTGase have sparked the surge of interests in food industries to replace animal TGase to perform similar biochemical properties to animal and plant TGase despite differences in amino acid composition (Luciano and Arntfield 2012). However, activation of mTGase requires removal of pro-peptide, which folds into an α-helix that covers the enzyme active site. The pro-peptide was reported to be vital for enzyme folding and inhibits enzyme activation within cells (Rickert et al. 2016). Activation of mTGase can be achieved via transglutaminase-activating metalloprotease (TAMEP) to expose the cysteine residue in the active site (Rachel and Pelletier 2013). As aforementioned, TGase recognizes glutamine and lysine residues for cross-linking of proteins. However, the enzyme does not exhibit any preferences toward a specific peptide sequence, which results in non-specific cross-linking of proteins (Rachel and Pelletier 2013). This attribute is beneficial in food industries to cross-link random proteins for desired products, but not in pharmaceutical applications where specific cross-linking is required. Efforts have therefore been invested to improve the specificity of TGase by screening preferred substrate peptides for wild-type TGase (Ohtsuka et al. 2000; Steffen et al. 2017; Sugimura et al. 2006).

18.3.3 Formylglycine-Generating Enzyme

Formylglycine-generating enzyme (FGE) is also known as sulfatase-modifying factor 1, which catalyzes the conversion of conserved cysteine residues in sulfatases to formylglycine (fGly). The latter residue is produced via co- or posttranslational modification of a conserved cysteine residue, which is located within the sulfatase motif. Since many organisms have endogenous FGE activity, this enzyme could be engineered for site-specific labeling of proteins (Carrico et al. 2007; Landgrebe et al. 2003). FGE (EC 1.8.99) is an oxidase that uses molecular oxygen in the oxidation of cysteine residues within the conserved sequence CXPXR of eukaryotes (Dierks et al. 1997), where X is usually serine, threonine, alanine, or glycine (Carrico et al. 2007), as referred to Fig. 18.3. In prokaryotes, however, it was reported that either a cysteine within sequence CxPxR or serine within the sequence SXPXR could be oxidized to form fGly (Carlson et al. 2008). The first prokaryotic FGE was identified in *M. tuberculosis* genome, which encodes only one type of functional FGE (Carlson et al. 2008). Since this peptide sequence is recognized by FGE, it could serve as a potential aldehyde tag for protein conjugation. By cloning the gene sequence of the motif as a fusion to the protein sequence, the peptide could be expressed along with the protein of interest, allowing the system to later be modified into aldehyde by FGE for protein conjugation. The potential of FGE in the conversion of the cysteine motif into fGly has been exploited in the development of ADCs (York et al. 2016). It has been shown that the location (C-terminal or N-terminal) of the tag will not affect the cysteine-aldehyde conversion (Carrico et al. 2007). Also, the location of the aldehyde tag was shown not to affect the stability and antitumor activity of the antibody conjugates (Drake et al. 2014). The installed aldehyde could then react with aminooxy or hydrazide reagents to form corresponding oxime and hydrazine conjugates (Appel and Bertozzi 2014). This FGE/aldehyde tag conjugation technology has been developed by Redwood Bioscience and Catalent Pharma Solutions to produce programmable, site-specific bioconjugates including ADCs (York et al. 2016).
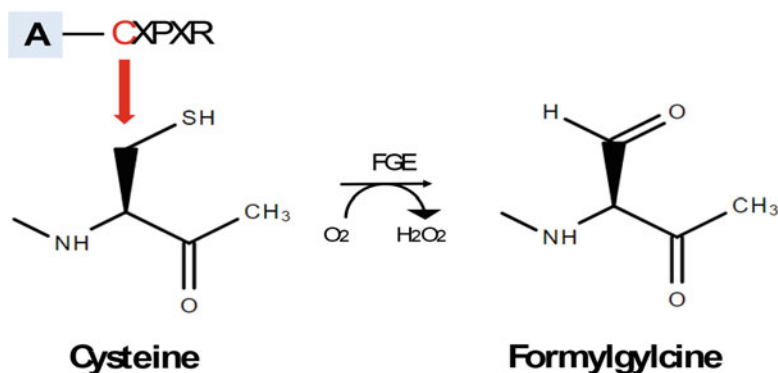
Fig. 18.3 FGE oxidizes the cysteine residue in CXPXR motif of protein of interest into formylglycine (fGly). The fGly bearing an aldehyde group which is ready for subsequent chemical conjugation

18.4   Potential Applications of Chemoenzymatic-Based Bioconjugation

Protein labeling is an important segment of research to study cellular processes, functions, and spatiotemporal dynamics. Critical information could be retrieved to identify the onset of diseases and to aid in drug development programs (Alberts et al. 2002). Conventional chemical conjugation in antibodies with the desired molecules has led to a heterogeneous mixture of antibody conjugate species, especially in ADCs. This has eventually resulted in undesirable attributes, such as aggregation, toxicity, shorter half-life, and loss of functionality in ADCs (Sochaj et al. 2015; Ta et al. 2011). Enzymatic conjugation is an ideal alternative for antibody labeling due to the substrate specificity of enzyme. Fusion of the desired motif sequence to the protein of interest using recombinant DNA technology enables the tag to be co-expressed together with the protein. There are a lot of enzymes being reported that can be exploited for protein bioconjugation, such as biotin ligase (Roux et al. 2012; Sueda et al. 2011), sortase (Chen et al. 2016; Ismail and Lim 2016), transglutaminase (Dennler et al. 2014; Siegmund et al. 2015), formylglycine-generating enzyme (Drake et al. 2014; York et al. 2016), SpyLigase (Fierer et al. 2014; Siegmund et al. 2016), farnesyltransferase (Dozier et al. 2014), phosphopantetheine transferase (Grünewald et al. 2015), and lipoic acid ligase (Cohen et al. 2012). In this chapter, the focus will be on the applications of sortase, transglutaminase, and formylglycine-generating enzyme in the biomedical field.

18.4.1   Potentials in Diagnostic

Chemoenzymatic conjugation for antibody labeling is a promising alternative in diagnostic due to the high specificity of the enzymes. Generally, an antibody is conjugated to different desired molecules for imaging or labeling purpose. SrtA has provided an established conjugation platform based on transpeptidation reaction by recognizing LPXTG motif. Sakamoto et al. (2010) reported the conjugation of LPETG-tagged ZZ domain of antibodies (antibody-binding domain) with enzymes, such as alkaline phosphatase (AP), luciferase (Luc), and glucose oxidase (GOD), to detect targeted molecules. Conjugation using SrtA, which retained the activity of both the ZZ domain and the conjugated enzymes, showed no impairment in their functions. Antibody conjugates could also be constructed easily in order to apply in enzyme-linked immunosorbent assay, which will make the detection to be more convenient. Signal could be detected once the antibodies bound without the need of additional incubation with secondary HRP-labeled antibodies (Sakamoto et al. 2010). A similar strategy was also applied to conjugate antibodies to live cells. In this context, glycine-rich peptides were first introduced to the cell surface to act as a SrtA substrate. Then, the incubation of glycine-tagged cells with LPETG-tagged single-chain fragment variable (scFv) antibodies yielded antibody-live cell conjugates for molecular imaging and cell homing applications (Ta et al. 2011). Levary and his group of researchers put SrtA to a challenge by conjugating ten pairs

of protein domains using the similar conjugation strategy. Each of the A33 antigen and IgG antibody was tagged with LPETGX recognition motif to react with five different triglycine-tagged proteins (green fluorescent protein, Fab antibody, gelonin, and albumin). They managed to achieve about 80% of fusion proteins with a retained native functionality (Levary et al. 2011). Recent publication reported the application of SrtA in noninvasive imaging of innate immune response. The C-terminal of camelid variable domains (VHHs) was engineered with LPETGGG for attachment of positron emission tomography (PET) radioisotopes. These imaging agents were reported to be easily produced using SrtA to cater diverse multimodality imaging agents (McCracken and Radu 2015). Another interesting work relating to SrtA was the development of invertase-based immunoassay using a personal glucose meter (PGM) for in-house antibody-antigen interaction detection. SrtA enzyme was used to conjugate scFv to the extracellular invertase to develop an invertase-based immunoassay. The conversion rate of sucrose to glucose upon incubation with scFv-invertase conjugate was monitored by using a personal glucose meter. This conjugate also allowed seamless swapping of other recombinant antibodies for detection of other diseases (Ismail and Lim 2016). Early work of TGases was performed on the biotinylation of antibodies. Two species of activated biotin served as acyl acceptors, whereas glutamine residues of a monoclonal antibody (mAb) against 2,4-dichlorophenoxyacetic acid (2,4-D) served as the acyl donor. Incubation of biotins with antibodies in the presence of TGase has successfully yielded biotinylated antibodies with a ratio of 1.1–1.9 biotins per antibody as revealed by mass spectrometry. ELISA results demonstrated the specific binding of biotin-conjugated antibodies to the avidin partner, and it gave comparable detection limit in the antigen-binding assay (Josten et al. 2000). Another group of researchers used TGases to produce radioimmunoconjugates with low off-target accumulation of radioactivity. The glycan, which is located at position 295 of the Fc, was first removed with PNGase F to expose the site for reaction with radioactive substrates (^{67}Ga). The incubation with TGase that yielded homogeneous radioimmunoconjugates was revealed by positron emission tomography. Also, an improved target-to-nontarget ratio was observed for TGase-conjugated radioimmunoconjugates (Jeger et al. 2010). In addition, TGases were also used to improve the attributes of antibodies by conjugation of a 20 kDa polyethylene glycol (PEG) to the glycine and lysine residues, which are located at positions of 101 and 164, respectively. PEGylated interferon-α isomers showed a better yield and purification as well as protein conformation, antiviral activity, and pharmacokinetics. Apart from that, PEGylated interferon-α isomers demonstrated a better antiviral activity and longer half-lives compared to unconjugated interferon-α isomers (Spolaore et al. 2016). Not much information was reported on the FGE-mediated antibody conjugation. The recent publication reported about the generation of high-titer aldehyde-tagged antibodies by supplementing copper (II) sulfate. Five amino acids (CXPXR) that are recognizable by FGE were fused with the protein of interest. The Cys residue within the sequence is converted into fGly residue which bears aldehyde groups during protein expression, and these aldehyde groups serve as potential groups for subsequent conjugation (York et al. 2016).

18.4.2 Potentials in Therapeutics

Antibodies have been established as a new therapeutic agent due to their high specificity against pathogens, making them relatively safe to treat various diseases (Dimitrov 2010). The evolution of antibodies over the years was made possible with the aid of antibody engineering and recombinant DNA technology. It has allowed customization of antibodies for specific pre-defined applications in therapeutics. Antibody-drug conjugates, therefore, emerge as one of the most promising classes of antibody-based therapeutic tools, which made use of the antibody specificity to deliver payloads to a target cell, and eliminate the bystander effect of conventional treatments (Akkapeddi et al. 2016). Previous publications demonstrated the conjugation of antibodies with various payloads using SrtA in the generation of ADCs. Modification was initially conducted at the C-terminal of antibodies by the addition of SrtA recognition motif LPETG to conjugate with the pentaglycine peptide-tagged substrates, such as monomethyl auristatin E (MMAE) and maytansine. It was reported that ADCs generated using SrtA have similar in vitro cell-killing activities with conventional conjugates (Beerli et al. 2015). However, toxic payloads often involve biosafety concerns, making them cumbersome for production. The production of toxic fused antibodies is always time-consuming and resulted in low yield due to the protein refolding. Kornberger and Skerra (2014) demonstrated a convenient way to generate an immunotoxin via SrtA. The fragment antigen-binding (Fab) antibody targeting Her2 was conjugated with a plant toxin, gelonin, aided by SrtA. SrtA recognition motif LPETG was introduced at the C-terminal of the Fab heavy chain, while the toxin was tagged with Gly_2 sequence at the N-terminal. This conjugation method allowed the toxic payload to be introduced in a controlled manner and devoid of mandatory biosafety levels for the generation of corresponding fusion proteins (Kornberger and Skerra 2014). Apart from the usual conjugation in therapeutics, SrtA was also applied in the production of bispecific antibodies. In this context, Wagner and his research group fused two full-sized IgG antibodies to form an IgG antibody heterodimer at the C-C terminal using a combination of Srt transpeptidation and click chemistry. The two antibodies were first labeled with either azide or DIBAC click peptide using SrtA. Subsequently, the fusion of the two antibodies was carried out via click chemistry between the peptides. This strategy requires no additional mutations within the antibody, which enables the native function of antibody to be retained without compromising the stability (Wagner et al. 2014).

Antibody-antigen construct has been reported to be used to shuttle antigens to dendritic cells (DC) to enhance antigen presentation for improving the antigen-specific T cell responses (Caminschi et al. 2009). The conventional methods to generate these antibody-antigen constructs are via chemical conjugation by targeting lysine or cysteine residues and recombinant technology by fusing the payload of interest. However, the chemical conjugation occurs randomly in most cases. In addition, the expression and protein purification of fusion proteins are laborious (Swee et al. 2013). To overcome these setbacks, Swee and co-workers (2013)

developed an antibody-antigen construct via an efficient and straightforward chemoenzymatic conjugation using SrtA. The heavy chain of the antibody (αDEC205) was genetically modified to introduce Srt recognition motif, LPETG. On the other hand, the antigen of interest (MHV-68) was tagged with a glycine-rich peptide. They concluded a tenfold reduction in viral load after the animals were immunized with the antibody-antigen construct (Swee et al. 2013). A similar work was also conducted by Duarte et al. (2016) using a single-domain antibody fragment (VHHs). The Srt recognition motif was introduced at the C-terminal of the VHH for conjugation with various antigen payloads. TGase is another workhorse for chemoenzymatic conjugation of antibodies to produce homogeneous ADCs (Dorywalska et al. 2015; Farias et al. 2014; Strop et al. 2016). However, TGases do not show any preferences toward specific peptide sequences. Glutamine and lysine residues could be the substrates for TGases as long as they are accessible to TGases (Coussons et al. 1992). Conjugation of an antibody with various molecules using TGases could simply be achieved via introduction of a K-tag (lysine residue) and Q-tag (glutamine residues), which are recognized by TGases (Kamiya et al. 2003; Lee et al. 2013; Lin and Ting 2006). However, researchers observed that TGases do not recognize naturally occurring glutamines in constant regions of glycosylated antibodies but only aglycosylated or deglycosylated antibodies (Jeger et al. 2010; Mindt et al. 2007; Strop et al. 2012). Hence, alternatives were developed to label substrate with the engineered glutamine tag (Strop et al. 2013) or deglycosylated glutamine residues to allow TGases for conjugation with payloads (Jeger et al. 2010). Strop and co-workers engineered a glutamine tag (LLQG) to attach diverse compounds at multiple positions of antibodies upon scanning the antibody constant domains. The researchers showed that the conjugation site significantly impacted ADC stability and pharmacokinetics. By introducing suitable amine linkers, TGases could therefore conjugate glutamine-tagged antibodies to various probes and drugs. Similar conjugation efficiencies were also observed for all IgG subtypes and even other different antibodies (Strop et al. 2013). On the other hand, Schibli and co-workers conjugated radionucleotides, such as ^{89}Zr and ^{67}Ga, to human IgG1 antibodies using mTGase. The glutamine residue (Q295), which is located in the Fc region of antibodies, was first deglycosylated with the enzyme PNGase F, so that the residue is now accessible to TGases. As a single antibody, it is consisting of two identical Fc regions; each antibody will therefore have two accessible sites for TGases conjugation. They also managed to conjugate payload to the other site of antibody by mutating a glutamine residue. The antibody is therefore able to accommodate more payloads via this strategy (Jeger et al. 2010). The strategy was later applied to directly attach antimitotic toxin monomethyl auristatin E (MMAE) to an antibody. In this study, the researchers observed a one-step conjugation process whereby direct conjugation of MMAE to deglycosylated antibody at Q295 using mTGases yielded heterogeneous ADCs with drug-to-antibody ratio (DAR) of between 1.0 and 1.6, and they required 80 molar excess of drug. On the other hand, two-step chemoenzymatic conjugation required only 2.5 molar excess of MMAE for conjugation and yet produced homogeneous ADCs with a DAR of 2.0. This two-step chemoenzymatic conjugation

involved attachment of a bioorthogonal linker to the deglycosylated antibodies in the first step using mTGases. Then, the modified MMAE was conjugated to the linker via chemical reaction (Dennler et al. 2014). FGE is recently applied in therapeutics. FGE adopts different working mechanisms from Srt and TGases by converting the cysteine (Cys) residue within CXPXR sequence into fGly residue, thereby generating an aldehyde tag for a conjugation purpose. By using this mechanism, Drake and co-workers cloned the FGE recognition sequence where the Cys residue was converted into fGly residue. Later, the aldehyde tag bearing antibodies were reacted with a hydrazine-iso-Pictet-Spengler (HIPS) linker and payload to generate ADCs via the formation of a covalent C-C bond (Drake et al. 2014). FGE was also being reported to aid in glycosylation of crystallizable fragment (Fc) of IgG1. By replacing the Fc N-glycosylation sequence with FGE consensus motif, the Cys residue was converted into fGly residue in the present of FGE. The glycan was then conjugated to aldehyde-labeled Fc to yield glycosylated Fc glycoforms without depending on natural protein glycosylation machineries (Smith et al. 2014).

18.4.3 Advantages and Disadvantages

Chemoenzymatic conjugation has shown to be a convenient approach to generate antibody conjugates for several applications either for diagnostic or therapeutic applications. No further modification in natural amino acid residues is required for the chemoenzymatic bioconjugation to occur. Enzyme-recognizable sequence can be cloned in directly with the protein of interest for co-expression prior to conjugation (Agarwal and Bertozzi 2015; Appel and Bertozzi 2014). Recognition motif can be fused easily with the target protein by using recombinant technology. In some cases, recognition motif can reside on either the antibody or conjugation partner depending on the applications (Dennler et al. 2015). Incorporation of enzyme-recognizable motifs via chemoenzymatic conjugation allows the production of homogenous ADCs as compared to conventional chemical conjugation strategies. This is due to the precise control over DAR and conjugation sites of payloads or other molecules for desired applications (Sochaj et al. 2015; Tsuchikama and An 2016). Enzyme-recognizable motifs are usually short (about 5–6 amino acids); hence issues regarding protein expression and purification could be minimized, and adverse immunogenic complications could be avoided (Hagemeyer et al. 2015). What is more attractive, enzyme-mediated conjugation is reported to be robust and flexible and does not interfere with antigen binding (Kamiya and Mori 2015; Swee et al. 2013; Wu et al. 2009). Moreover, the conjugation reaction using enzyme is mild, which is able to preserve the functionality of both the antibodies and conjugation partners (Strop 2014).

Despite the advantages portrait by enzymatic bioconjugation of antibodies, the existence of disadvantages is inevitable. For example, Srt conjugation is reversible despite its high specificity as the left over glycine residue in the first step can act as a nucleophile to reform the original species. Its reversible nature has restrained the conjugation efficiency and its use (Rashidian et al. 2013). This also explains the need

of higher concentrations of Srt for conjugation which defines another drawback of Srt in conjugation (Chen et al. 2011). However, researchers reported that by replacing the amide bond between threonine and glycine with an ester within LPXTG, the peptide is unable to undergo reversible reaction efficiently and remained as an enzyme substrate. Almost 100% conversion to product was successfully obtained using this approach (Williamson et al. 2012). Another setback of Srt conjugation approach is the slow reaction kinetics. To compensate for the poor reaction kinetics, reaction ranging from 1 to 3 h or even overnight incubation is often required to obtain high conjugation yields (Chen et al. 2011). Mutagenesis and maturation studies were reported to improve the reaction kinetics up to 140-fold by using yeast display technology (Chen et al. 2011). In addition, broad substrate specificity of TGases has significant importance in food and textile industries to catalyze amide bond formation (Gundersen et al. 2014). However, this attribute appears to be a double-edged sword for TGases due to the cross-reaction with nontarget substrates and therefore impedes their applications in biotechnological fields (Steffen et al. 2017; Strop 2014). Efforts have been put in over the years to screen for improvement of TGases substrate specificity using biopanning (Sugimura et al. 2008) or computational modeling approach (Yokoyama et al. 2010) to obtain the best sequence or motif, which is able to be recognized by TGases. The other potential downside of chemoenzymatic conjugation is provoking undesired immune response in humans due to the immunogenicity of the peptide sequences introduced (Agarwal and Bertozzi 2015). Also, chemoenzymatic bioconjugation is usually limited only to the N- or C-terminal, which refrains its applications, if conjugation within antibodies is desired or both terminals are vital for protein functionality (Carrico et al. 2007; Theile et al. 2013). However, the possibility of using hybrid protocols incorporating enzymatic and chemical conjugation methods has helped to overcome some of these bottlenecks.

18.5 Current and Future Trends of Chemoenzymatic Bioconjugation

Monoclonal antibodies are a growing class of therapeutic agents, and they are predominantly found in various diagnostics. Despite excellent results proven in biopharmaceuticals, unconjugated antibodies still suffer from various setbacks, which restrict their wide applications (Dennler et al. 2015). Antibody conjugation has been reported to improve antibody attributes, such as functionality, pharmacokinetic, therapeutic index, solubility, and half-life (Badescu et al. 2014; Jevševar et al. 2012; Junutula et al. 2008). Various chemoenzymatic bioconjugation methods are available to generate better antibody conjugates for more applications. However, considerations need to be taken into perspective when performing antibody conjugation, such as enzyme specificity, tag size, tag location, incorporation kinetics, and modification sites (Rashidian et al. 2013). As chemoenzymatic bioconjugation has matured over the years, the number of techniques available for conjugation has multiplied. Many more chemoenzymatic conjugates are advancing to clinical trials

(McLaughlin and LoRusso 2016) or are heavily applied in diagnostics (Ismail and Lim 2016; Spolaore et al. 2016). However, much work remains to be done so that these conjugation technologies could be applied widely. Advancement of molecular technologies and protein engineering will be a driving force for researchers to address the current setbacks that portrayed by the current chemoenzymatic bioconjugation methods. In the near future, we envision chemoenzymatic bioconjugation to have the capacity and potential to be tailor made for specific purposes, and more enzymes with better attributes will be discovered for biomedical applications. Chemoenzymatic conjugation is foreseen to play a major role in innovative science, such as antibody-DNA conjugation for detection or formation of nanostructure for drug delivery or diagnostic tools. The potential of chemoenzymatic conjugation should not be only restrained for ADCs but incorporate at all levels of science to attain suitable alternatives for problems.

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