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PII S1050-1738(12)00213-7 **TCM**

Enzymatic Antibody Tagging: Toward a Universal Biocompatible Targeting Tool

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Targeted delivery or "smart delivery" of pharmaceutical or imaging agents and even entire cells such as stem cells is an emerging trend in modern biotechnology. A binding ligand such as an antibody that can specifically bind to receptors expressed at a disease site is an essential component of such constructs. Different chemical methods have been widely used to apply antibodies for delivery systems; however, they typically result in impairment or loss of antibody functionality. Enzyme-mediated conjugation approaches have been developed to overcome this major disadvantage of conventional chemical methods. Sortase, an enzyme derived from Staphylococcus aureus, is able to provide a biochemically robust, highly reproducible, and site-specific coupling method for the conjugation of antibodies to pharmaceutical agents, nanoparticles, and cells for drug delivery, molecular imaging, and cell homing. Here, we review the use of sortase and other enzyme-based methods as bioconjugation tools with a focus on cardiovascular applications. (Trends Cardiovasc Med 2012;22:105-111)

• Introduction

Antibody tagging is one of the essential methods to achieve targeted molecular imaging and targeted drug or regenerative cell delivery for the diagnosis and treatment of diseases. Targeted drug delivery can improve treatment efficacy of a variety of conditions, such as cancer, diabetes, and cardiovascular and inflammatory diseases. Although the treatment and imaging of cancerous tumors is the most widely studied application of targeted delivery, this approach has gained increasing interest in other areas such as cardiovascular diseases. Cardiovascular disease (CVD) is a major health issue and the leading cause of mortality and morbidity worldwide (Roger et al. 2011). Therefore, there is an urgent need to develop better diagnostic imaging agents for vulnerable plaque detection, for example, as well as more efficient treatment options such as stem cell delivery for cardiac repair. The key to achieving these objectives lies in more effective use of medication that can be targeted directly to the diseased tissue. Targeting drugs to desired areas not only enhances the potency of the drugs but also minimizes side effects because the injected dose is greatly reduced.

Conventionally, targeted delivery is achieved via chemical conjugation of

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Published online 11 August, 2012.

1050-1738/$-see front matter

medication to specific targeting ligands such as peptides or antibodies. However, there are many drawbacks associated with the use of this methodology, and they are reviewed briefly in the next section. Emerging enzyme-mediated coupling methods are advantageous compared to conventional approaches because they are highly site specific, robust, reproducible, rapid, and mild, ensuring retention of antigen-binding activity of the targeting ligand. This review focuses on one of the most interesting enzymes in this area, sortase, which is a transpeptidase produced by gram-positive bacteria to covalently anchor cell surface proteins to the cell wall. Sortase has been extensively studied for a range of biotechnology applications, and it has recently been employed to attach small recombinant antibodies to fusion partners for targeting purposes in CVDs (Ta et al. 2011). Here, we review the versatile applications of this enzyme, especially in CVD, and compare the use of sortase to other enzymes reported for protein or antibody tagging.

• Advantages and Disadvantages of Chemical Conjugation Methods

Using cross-linking agents has been the most common method for covalently linking pharmaceutical agents to antibodies for targeted delivery. A number of functional groups, such as amine, thiol, and carboxyl groups, attached to either the therapeutic agents or the antibody molecules are widely used for chemical conjugation (Hermanson 2008). Cross-linking reactions between these groups can be performed by the use of special bispecific chemical reagents. Because these reactive groups are usually present in abundance in targeting ligands such as antibodies, reaction times are generally short. Bispecific cross-linkers provide flexible, simple, and fast approaches for conjugation.

Most conjugation methods have targeted natural reactive groups of endogenous amino acids that are commonly distributed throughout the antibody structure. If the conjugation involves critical residues essential for antigen binding, the functionality of antibodies, particularly small recombinant antibody fragments, is impaired. To overcome this problem, carbohydrate chains typically attached to the constant heavy-chain domain within the crystallizable fragment region of the antibody were used (Manjappa et al. 2011). However, this method is limited to glycosylated antibodies. Other disadvantages include the formation of multicomponent heterogeneous mixtures, low reproducibility, harsh reaction conditions, and the need for pure protein samples to avoid coupling of contaminating proteins.

Major efforts have been invested to develop site-specific conjugation via chemical methods. These are based on the introduction of unique functional groups that are not present in natural amino acids such as ketones and azides into proteins by chemical modification of the N-terminus of the protein (Gilmore et al. 2006). Other methods also include unnatural amino acid mutagenesis (Wang and Schultz 2004) or the use of enzymes that transfer prosthetic groups to proteins (Chen et al. 2005). However, these methods face practical limitations in terms of feasibility, scalability, and efficacy (Wu et al. 2009). Site-specific conjugation can also be achieved via an affinity tag (eg, protein fusions with histidine [His] tags [Schmid et al. 1997]), but this noncovalent and nonbiocompatible method is unsuitable for human use. Alternative chemical bioconjugation methods such as native chemical ligation (Tam et al. 2001) and intein-based ligation (Iakovenko et al. 2000) have been reported. Although these methods are specific, they have common limitations, including the difficulty of synthesizing peptide thioesters and large tags that can lead to expression problems (Nyanguile et al. 2003).

Despite these drawbacks, chemical conjugation has resulted in two successful antibody-drug conjugates. Brentuximab vedotin (SGN-35), approved by the U.S. Food and Drug Administration in August 2011 for the treatment of lymphoma (Gualberto 2012), was created by the partial reduction of the antibody disulfides and subsequent alkylation of the reduced antibodies with maleimido-containing drugs (Doronina et al. 2003). Trastuzumab emtansine (T-DM1), a herceptin-drug conjugate for the treatment of breast cancer, is currently in phase III clinical trials (Roche 2012). Key steps in the production of T-DM1 included chemical linkage of a maleimido-containing linker molecule to antibody through lysine residues, followed by reacting the free sulfhydryl on the drug with the linker-antibody intermediate (Krop et al. 2010).

• Genetic Antibody Fusion

Genetic fusion of antibodies to therapeutic proteins and peptides has initially been used to overcome the limitations of chemical conjugation. Using recombinant technology, various antibody-therapeutics such as fibrin or platelet targeted thrombolytic and anticoagulants have been developed mainly for the prevention or treatment of thrombosis (Hagemeyer et al. 2004a, Hagemeyer et al. 2004b, Peter et al. 2000a, Peter et al. 2000b, Topcic et al. 2011). This method provides site-specific ligation, and the fusion proteins can generally be produced with preserved activity in large quantities at low cost in *Escherichia coli* (Hagemeyer et al. 2004b). However, genetic fusion has several limitations, including the inability to conjugate antibodies directly to particles or live cells and the inability to modify the ratio between the antibody and therapeutic protein.

• Sortase-Mediated Antibody Tagging

The Concept

Sortase, a calcium-dependent enzyme, was first characterized and purified from *Staphylococcus aureus* extract by Ton-That et al (1999). Surface proteins of *S. aureus* (gram-positive bacteria) are anchored to the bacterial cell wall by sortase, a transpeptidase enzyme that cleaves polypeptides between the threonine and the glycine of the small LPXTG motif, where X denotes any amino acid (Ton-That et al. 1999). The liberated carboxyl group of threonine subsequently forms an amide bond with the amino group of the pentaglycine on bacterial peptidoglycan, thereby linking the surface proteins to the bacterial wall. The sulfhydryl (cysteine) group of sortase is necessary for its activity and may function as a nucleophile at the peptide bond between threonine and glycine (Ton-That et al. 1999). Sortase was also reported to mediate the coupling between -LPETG-COOH and alkynamine (Parthasarathy et al. 2007, Ta et al.

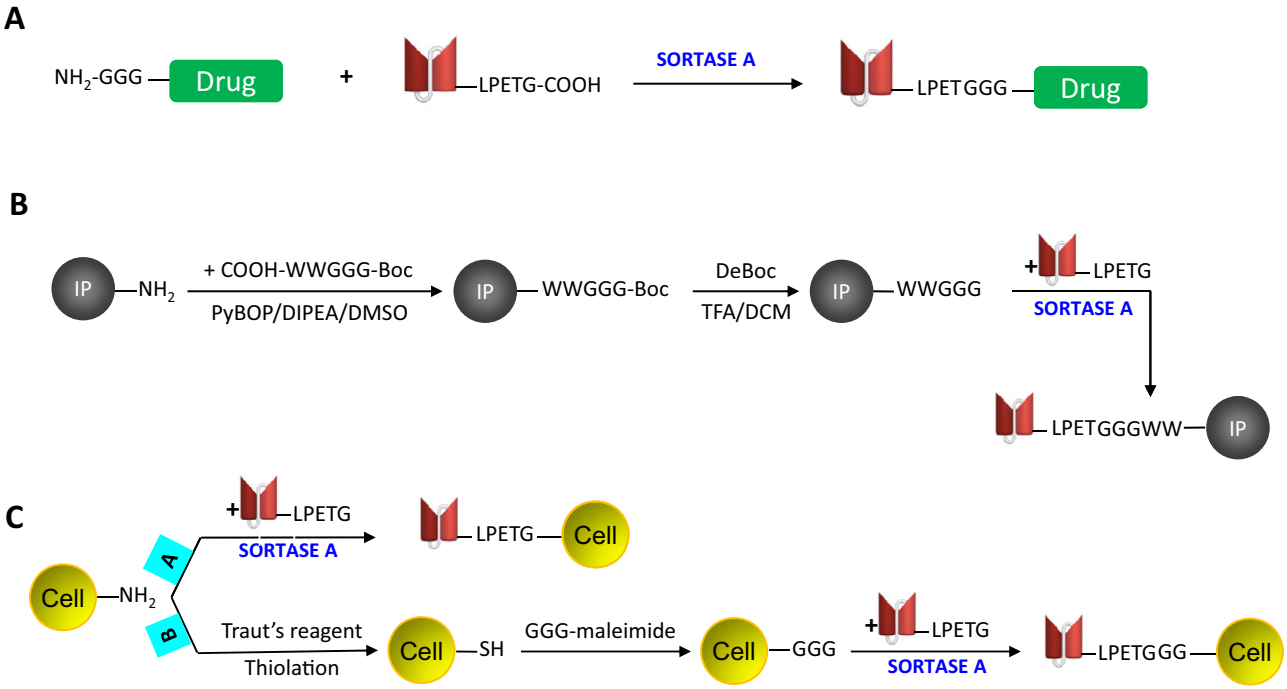

Figure 1. Illustration of the coupling procedures of drugs, imaging particles, and cells to single-chain antibodies. (**A**) Drug: Sortase catalyzing a condensation reaction between a C-terminal -LPETG tag on the antibody and a triglycine (GGG) handle on the pharmaceutical protein or small drug molecule. (**B**) Imaging particle (IP): *tert*-butyloxycarbonyl–protected GGGWW peptides were introduced onto amine particles via carboxyl activation. After deprotection, a sortase-mediated condensation reaction between antibody-LPETG and GGG particles was performed. (**C**) Cell: Protocol A comprises the sortase-catalyzed reaction between antibody-LPETG and amine groups of the cell surface. Protocol B includes the thiolation of cell membrane amine groups (sulfhydryl addition), the introduction of GGG-maleimide on the cell surface via the reaction between the sulfhydryl (–SH) groups and the maleimide groups, and the covalent coupling between antibody-LPETG and GGG groups on the cell membrane via the sortase-mediated reaction. Adapted from Ta et al. (2011).

2011). It was thought possible that free amine groups could function as a nucleophile for the enzyme; however, alkylamine was less reactive than GGG or Gly_n and did not result in high conjugation yield.

Applications

Since its discovery and isolation from *S. aureus*, sortase has been used primarily as an engineering tool. Scientists have employed the enzyme to immobilize proteins to solid surfaces such as polystyrene beads (Parthasarathy et al. 2007), biosensor chips (Clow et al. 2008), and microspheres (Wu and Proft 2010). Generally, these solid surfaces were chemically modified with either alkynamine or sortase ligand (triglycine linker peptide: Gly-Gly-Gly). The proteins were fused with a C-terminal LPETG SrtA recognition motif via polymerase chain reaction cloning. The ligation between -LPETG and GGG- motifs is then mediated by the enzyme under mild physiological conditions. Similarly, using these two sortase recognition motifs, the enzyme was used for synthesis of neoglycoconjugates (Samantaray et al. 2008), oligomerization of protein/peptide–protein/peptide (Parthasarathy et al. 2007), and labeling of cell surface proteins on cells (Tanaka et al. 2008). Intracellular sortagging was also performed for protein ligation in living cells (Strijbis et al. 2012). In this study, calcium-independent sortase from *Streptococcus pyogenes* was employed instead of the calcium-dependent enzyme from *S. aureus*. Intracellular protein ligation was achieved in *Saccharomyces cerevisiae* and in mammalian HEK293T cells, both in the cytosol and in the lumen of the endoplasmic reticulum, enabling secretion of the reaction products.

Applications in CVD

Recently, sortase has been used to conjugate a single-chain antibody (scFv) directed against activated platelets to nanoparticles, cells for molecular imaging and cell homing in CVDs (Ta et al. 2011). This scFv specifically binds to a ligand-induced binding site (LIBS) on a highly expressed glycoprotein GPIIb/IIIa receptor on the surface of activated platelets, which play a pivotal role in thrombosis, atherosclerosis, and inflammation (Stoll et al. 2007). The possibility of generating scFvs via phage display against epitopes that mark specific functional states of cells makes this antibody format attractive as a molecular tool particularly for targeting (Eisenhardt et al. 2007a, Eisenhardt et al. 2007b, Schwarz et al. 2004, Schwarz et al. 2006). Thus, developing tagging methods for these scFvs is highly attractive (Hagemeyer et al. 2009). Iron oxide microparticles, which have been used successfully for magnetic resonance imaging of thrombosis (von zur Muhlen et al. 2008a) and inflammation (von zur Muhlen et al. 2008b), were used in this study along with cell homing to thrombosis. Generally, the conjugation procedure involves two stages: (1) introducing the GGG peptides (as sortase nucleophiles) to the surface of magnetic particles and cells by a series of chemical reactions and (2) incubating triglycine-tagged components with sortase enzyme and anti-LIBS scFv (Figure 1). The conjugation efficiency ranged from 50% to

70%, and bioactivity of the scFv after coupling was preserved. Targeting of scFv-coupled cells and nanoparticles to activated platelets was strong and specific in vitro and in vivo. Since then, sortase has been utilized to catalyze the fusion of LPXTG-tagged anti-epidermal growth factor receptor single-chain antibody (sc528) and GGG-biotin (Madej et al. 2012). This biotechnological approach provides a versatile and broadly applicable tool that allows for targeted drug and regenerative cell therapy and targeted molecular imaging in cardiovascular and inflammatory diseases and beyond.

Advantages and Disadvantages

The sortase-based method provides a robust and mild approach for site-directed, covalent coupling of proteins. This method requires the use of two short amino acid motifs (LPETG and GGG) that are easily incorporated into substrates of interest either by chemical synthesis or by genetic engineering. The small size of the fusion tags is advantageous because they have minimal effect on the activity of the target protein or scFv antibody. There are also fewer issues associated with expression and purification. LPETG motif can be introduced in anti-LIBS platelet antibody at the C-terminus by polymerase chain reaction cloning while preserving the functionality of the scFv (Ta et al. 2011). Because the C-terminal end is not within the targeting regions of the scFv, the likelihood of compromising the antigen-binding region is minimized (Hagemeyer et al. 2007). Notably, the high specificity of sortase allows coupling of crude protein preparation, thereby shortening protein purification protocols while preventing ligation of contaminants (Ton-That et al. 1999). Furthermore, sortase is easy to produce in large amounts, making this method very cost-efficient (Clow et al. 2008). A current limitation of the method is the difficulty in controlling the amount of coupled proteins (Wu and Proft 2010). In addition, due to the nature of the sortase reaction, introduction of -LPETG and GGG- motifs to the target proteins is restricted to the C-terminus and N-terminus, respectively. Although the small size of the sortase tags minimizes the probability of immunogenic reactions, there is no evidence for the safety, or lack thereof, of these tags in vivo. Another disadvantage is the slow kinetics of the sortase reaction, which can in some cases require a relatively long (overnight) incubation period to achieve a good conjugation yield (Clow et al. 2008). However, it was recently shown that sortase activity could be improved up to 140 times using a directed evolution strategy involving yeast display (Chen et al. 2011).

- **Other Enzyme-Based Ligation Approaches**

Transglutaminase

Transglutaminases (TGases) catalyze the posttranslational modification of proteins, the formation of amide bonds between the **γ**-carboxyamide group of an intact protein-bound glutamine (Gln or Q) and a variety of primary amines, particularly the **ε**-amino group of lysine (Lys or K) (Lin and Ting 2006). They are ubiquitous in multicellular organisms and function in different biological events (Lin and Ting 2006). Briefly, short peptide sequences with Q residues—Q-tags, such as PKP**QQ**FM (Taki et al. 2004) and G**QQQ**LG (Hu and Messersmith 2003)—with high specificity toward TGases were genetically introduced at the N-terminus of the target proteins for fusion with primary amine-containing substrates.

Applications

Guinea pig liver TGase (gpTGase), a calcium-dependent enzyme, has been used for site-specific fluorescent labeling and glycosylation of proteins (Sato et al. 2004, Taki et al. 2004). It has also been used to conjugate small-molecule probes to surface proteins of living mammalian cells (Lin and Ting 2006) and for immobilization of single-chain antibody (Sugimura et al. 2007). In addition to gpTGases, microbial transglutaminase (MTGase) from *Streptomyces mobaraenis* has also been widely employed due to its calcium independency, handling feasibility, availability of mass production by the microorganism (Tanaka et al. 2005), and lower substrate specificity for the amine acceptor site around the Gln residues (Sato et al. 2001). MTGase has been used for labeling of single-chain antibody (Takazawa et al. 2004) and for site-specific modification and PEGylation of different pharmaceutical proteins for clinical applications (Besheer et al. 2011, Tanaka et al. 2005).

Advantages and Disadvantages

Although TGase-mediated conjugation is highly selective and mild, and results in a highly stable cross-linked bond, TGase has low specificity for Q-tag, which could result in labeling non–Q-tagged proteins (Lin and Ting 2006). In addition, TGases have not been extensively investigated, and the specific determinants for the reaction are not well understood. Not all glutamine residues in the protein substrates are candidates for a TGase reaction (Fontana et al. 2008). As mentioned previously, by incorporating specific TGase-recognition peptide tags into proteins, TGase-mediated site-specific conjugation of proteins is possible if intrinsic Lys and Gln residues are not available in properly folded target proteins. However, the variation of peptide tags as substrates for TGases is currently limited because the substrate specificity of these enzymes has not been clearly established (Tanaka et al. 2005).

Biotin Ligase: AviTag Enzyme Technology

The concept

This approach takes advantage of the strong binding between biotin and avidin but avoids possible inactivation of proteins of interest by nonspecific or random biotinylation associated with chemical biotin labeling. Using cloning, the target protein is tagged with an amino acid sequence that is specifically biotinylated by biotin ligase. The biotin-labeled protein subsequently interacts with (strept)avidin-bearing subjects and forms the desired conjugates. Biotin ligase (BirA), an enzyme derived from *E. coli*, is capable of catalyzing amide linkage between a biotin and a specific lysine side chain within a 15-amino acid acceptor peptide (AP) sequence or a so-called AviTag in the presence of ATP (Schatz 1998). In *E. coli*, only one protein is biotinylated—the biotin carboxyl carrier protein (BCCP) (Choi-Rhee et al. 2004). The AviTag sequence with only 15 amino acids (GLNDIFEAQKIEWHE) contains the optimal peptide sequence reported to be biotinylated by BirA (Beckett et al. 1999). AviTag can be cloned into either the N-terminal (Chen et al. 2005) or the

C- terminal (Sung et al. 2011) of a fusion protein, or at internal protein locations (Tannous et al. 2006), if the peptide domain forms a surface-exposed loop accessible to the BirA enzyme.

Applications

BirA has been employed for protein and single-chain antibody biotinylation both in vivo and in vitro (Maeda et al. 2008, Sung et al. 2011) and may also be used in animals (de Boer et al. 2003). It is specific in both mammalian cells and non-mammalian cells and is able to biotinylate the AP tag in different cellular compartments (at the cell surface, in the endoplasmic reticulum, in the cytosol, and in the nucleus) (Howarth and Ting 2008). The enzyme has been employed for many applications, such as imaging of proteins for trafficking studies (Howarth et al. 2005), purifying proteins and antibodies (Barat and Wu 2007), tracking cells and tumors in vivo (Tannous et al. 2006), purifying and targeting viral vectors to specific cells (Nesbeth et al. 2006), and immobilization of proteins (Min et al. 1999). Recently, it has been used for targeting imaging probes in living animal for in vivo vascular imaging (Bartelle et al. 2012) and for molecular imaging of thrombosis (Wang et al. 2012). In addition to BirA, endogenous mammalian biotin ligase (holocarboxylase synthetase) was also employed for this purpose (Tannous et al. 2006). However, this protocol involved a larger biotin acceptor peptide tag (129 amino acids), and its efficiency varied between cell types. Efficiency was enhanced more than 10-fold by overexpressing BirA in these cells (Niers et al. 2011).

Advantages and disadvantages

Although biotin ligase has excellent peptide specificity, versatility is limited because the enzyme is extremely specific for the biotin structure; consequently, labeling of biotinylated proteins can only occur with (strept)avidin-bearing subjects. Because (strept)avidin is large in size (56-69 kDa), it can possibly affect protein function (Howarth and Ting 2008). To overcome this, Chen et al. (2005) developed ketone-modified biotin molecules that are accepted by BirA as cofactors, allowing conjugation of ketone-tagged proteins with hydrazide- or hydroxylamine-functionalized molecules. However, this approach lacks sensitivity due to the kinetics of hydrazide labeling.

• Summary

Conventional chemical bioconjugation approaches utilizing reactive functional groups naturally present in all proteins are subject to a lack of specificity and occasionally harsh reaction conditions, which may negatively affect activity of the coupled proteins. Enzyme-based methodologies have overcome many problems associated with chemical ligation. Because they are highly specific and occur in mild physiological conditions, they can eliminate the possibility of protein inactivation. The transpeptidase sortase cleaves proteins between a threonine and a glycine residue within a conserved LPXTG motif near the C-terminus and covalently links it to the terminal amino group of the triglycine. Not only does it provide an efficient conjugation approach but also, more important, it retains the functionality of the targeting antibody component and avoids heterogeneous multilabeled mixtures. Coupling is highly specific due to the LPXTG and GGG unique motifs. In addition to sortase, transglutaminase and biotin ligase show promise for enzyme-based ligation, although transglutaminase is less specific than the other two methods and biotin ligase is more difficult to use than sortase due to its larger recognition tags.

• Acknowledgments

H.T.T., K.P., and C.E.H. are supported by a National Heart Foundation Postdoctoral Fellowship (PF09M4688), an Australian Research Council Future Fellowship (FT0992210), and a National Health and Medical Research Council Career Development Fellowship (472668), respectively.

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PII S1050-1738(12)00214-9 **TCM**

Erratum

Due to the journal *Trends in Cardiovascular Medicine* publishing off schedule, the article ***Fibroblast Growth Factor Homologous Factors in the Heart: A Potential Locus for Cardiac Arrhythmias*** (Eric Q. Wei, Adam S. Barnett, Geoffrey S. Pitt, Jessica A. Hennessey) was allocated to the late running Volume 21 Issue 7 (year 2011).

The paper was officially commissioned on 8 January 2012, accepted on 15 May 2012, and published on 3 August 2012. The Publisher recognises that this could be confusing for both the author and reader alike and sincerely apologises for any confusion or inconvenience caused.

PII S1050-1738(12)00323-4 **TCM**