Preparation of Chain-End Clickable Recombinant Protein and Its Bio-Orthogonal Modification

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Abstract:

Introducing unique functional group into protein is an attractive approach for site-selective protein modification applications. In this report, we systemically investigated four site-selective strategies to introduce azide functionality into recombinant thrombomodulin (TM₄₅₆), *via* direct recombinant expression with unnatural amino acid, chemical, and enzymatic modification for its bio-orthogonal modification application. First, a straightforward recombinant method to express TM₄₅₆ with azide functionality near C-terminus by replacing methionine with azidohomoalanine from methionine auxotroph *E. coli* cell was investigated. Next, a sortase-mediated ligation (SML) method to incorporate azide functionality into the C-terminus of recombinant TM₄₅₆ was demonstrated. The third is to add azide functionality to the N-terminal amine of recombinant TM₄₅₆ *via* amidation chemistry, and the fourth is tyrosine selective three-component Mannich reaction to introduce azide functionality to recombinant TM₄₅₆. Overall, SML of recombinant protein affords the highest overall yield for incorporating azide functionality into the C-terminus recombinant TM₄₅₆ since the key protein expression step uses natural amino acids. Also, single site modification facilitates the highest TM₄₅₆ activity.

Keywords: azide; bio-orthogonal modification; copper-free click chemistry; sortase A; thrombomodulin

1. Introduction

Modification of protein is a very important and necessary approach for studying protein structure and function, and the mechanisms of biological pathways that the protein is involved in. Furthermore, modification can expand protein's functional capacity, especially for therapeutic proteins for enhanced pharmacodynamic and pharmacokinetic properties. The key point for protein modification is to carry out a well-defined site-selective chemistry without reducing the protein's original activity and in high efficiency for generating homogeneous product. Bio-orthogonal chemistry facilitates site-selective modification by targeting to a specific functionality and has shown great potential for protein modifications of different interests. Azide has been proved as a versatile bio-orthogonal chemical reporter, which is small and inert in physiological settings and used for modifying diverse classes of biomolecules [1-5]. In particular, azide-based ligation reactions have been extensively explored for highly selective and biocompatible modification of proteins for biomedical applications [6-8]. Nowadays, three major bio-orthogonal reactions utilizing azide functionality have been fully developed, including Staudinger ligation, click chemistry and copper-free click chemistry (CFCC), among which CFCC is the most convenient and efficient one. Therefore, efficiently introducing azide functionality into proteins has become the key step and versatile approach for site-selective protein modification for both research and applications.

Thrombomodulin (TM) is a membrane glycoprotein mainly expressed by vascular endothelial cells as a major anticoagulation component [9]. TM consists of 557 amino acids divided into five distinct domains: *N*-terminal lectin-like domain (designated as TMD1); six epidermal growth factor (EGF)-like domain (TMD2); Ser/Thr-rich domain (TMD3); transmembrane domain (TMD4); and cytoplasmic tail domain (TMD5). The different domains are responsible for different biological functions of TM [10]. In the past decades,

various domains of TM have been cloned and expressed for TM structural and functional study [11]. Further, recombinant TMs of different domains show promising antithrombotic and anti-inflammatory activity in both rodents and primates models. A recombinant soluble TM has been approved for therapeutic application in Japan [12]. In addition, TM has been considered as an excellent candidate for biomedical engineering applications. Chaikof *et al.* reported a truncated TM construct with an azidohomoalanine near C-terminus for site-selective modification *via* Staudinger ligation [13] and oriented immobilization through click chemistry [2]. Besides, bioactive modification of pancreatic islets with the azide-containing TM *via* Staudinger ligation was investigated by the same group, showing the potential to reduce procoagulant and pro-inflammatory responses upon transplantation [14,15]. All these results demonstrated the feasibility of recombinant azide-TM for cell surface re-engineering and its promising application for cell transplantation. Recently, we proposed a recombinant TM-liposome conjugate as a bio-inspired antithrombotic agent, which mimics both the protein and lipid membrane structure of the native endothelial membrane TM [16]. Briefly, a TM of EGF-like 4-6 domains with an azidohomoalanine near C-terminus (rTM₄₅₆-N₃) was expressed *via* replacing methionine with azidohomoalanine from methionine auxotroph *E. coli* cell. Then, site-selective conjugation of the rTM₄₅₆-N₃ with liposome *via* Staudinger ligation and CFCC afforded recombinant TM₄₅₆-liposome conjugates, both of which showed enhanced protein C activation activity facilitated by lipid membrane.

Introducing unnatural amino acids into recombinant TM provides versatile tool for site-selective chemistry for protein modification applications. However, the low level of protein expression with unnatural amino acid limits its practical applications. Therefore, to find a way to introduce azide into protein efficiently and site-selectively is highly needed for all promising researches and applications of proteins like TM₄₅₆ described above. In this study, we systemically investigated recombinant, enzymatic and chemical strategies to introduce

azide functionality into TM₄₅₆ site-selectively at either *N*-terminus or *C*-terminus. First, we investigated recombinant expression of recombinant TM₄₅₆ with azide functionality near *C*-terminus by replacing methionine with azidohomoalanine from methionine auxotroph *E. coli* cell. Second, we expressed TM₄₅₆ with sortase A recognizing motif LPETG for introducing azide functionality into the *C*-terminus *via* sortase-mediated ligation (SML). Third, a chemical method was used to add azide molecule to the *N*-terminal amine of TM₄₅₆ *via* amidation chemistry. Finally, multiple azide functional groups were introduced into TM₄₅₆ *via* a tyrosine-selective three-component Mannich reaction (Figure 1). The azido-TM₄₅₆ derivatives obtained in all four methods were confirmed for site-selective modification and immobilization of recombinant TM through CFCC successfully.

Figure 1

2. Materials and methods

2.1. Materials and Reagents

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. pET 39b vector, pET 28b vector, competent cells, and kanamycin sulfate were purchased from EMD Chemicals (Philadelphia, PA). The mouse monoclonal antibody specific to human TM and Goat Anti-Mouse IgG H&L (FITC) were purchased from Abcam (Cambridge, MA). Human protein C, human thrombin and human antithrombin III were obtained from Haematologic Technologies Inc.. L-azidohomoalanine was from AnaSpec Inc. (Fremont, CA). Chromogenic thrombin substrate BIOPHEN-CS01 was obtained from Anisara. Anti-PEG monoclonal Ab E11 was purchased from Academia Sinica (Taipei, Taiwan). 4N-BOC-Gly-Gly-OH and N-hydroxysuccinimide were purchased

from Sigma-Aldrich (St.Louis, USA). DBCO-Cy5, Azido-PEG₃-Amine, Azido-PEG₄-NHS Ester and DBCO-PEG₅₀₀₀-OMe were purchased from Click-Chemistry Tools (Scottsdale, AZ).

2.2. Expression of FLAG-TM3

The gene encoding the EGF domain 4-6 of human thrombomodulin with a FLAG tag (FLAG-TM3) and a C-terminal LPETG motif was designed and synthesized (Genscript Inc.). In order to prevent oxidation and reduce proteolytic susceptibility, three residues of TM were mutated (M388L, R456G and H457Q) as described previously [17]. The FLAG-TM3 gene was then transferred into pET-28b vector containing a C-terminal His-tag between *NcoI* and *XhoI* sites to obtain the expression plasmid pET28b-FLAG-TM3.

The expression plasmid and pET28b-FLAG-TM3 was transformed into *E. coli*. B834 (DE3) cells for expression. The *E. coli*.B834 (DE3) cells containing the expression plasmids were incubated in LB medium with 35 mg/L kanamycin at 37 °C until an OD₆₀₀ of 0.8 was reached, and then IPTG was added to a final concentration of 1 mM to induce the overexpression of TM₄₅₆ by incubation for 5 hrs at 37 °C. The bacteria were then centrifuged at 8,000g for 5 min, and the cell pellet was collected and stored in -20 °C.

2.3. Expression of azido-FLAG-TM3

For azido-FLAG-TM3 expression, the *E. coli*.B834 (DE3) cells were incubated at 37 °C in M9AA medium (1 L) supplemented with 1 mM MgSO₄, 0.1 mM CaCl₂, 0.4 % (wt/v) glucose, 1mg/L thiamine chloride, 35 mg/L kanamycin and proteinogenic amino acids to the point that OD₆₀₀ value around 0.8. Methionine was then removed from the medium by centrifugation for 10 min at 4000 x g at room temperature, and the cell pellet was washed with M9AA medium. The pellet was resuspended in 1 L of the M9AA medium with all the

components mentioned above except methionine and further incubated at 37°C for 1 h to starve the cells. L-azidohomoalanine was then added to the incubation medium to a final concentration of 80 mg/L and after incubation for another 1 h, protein expression was induced by 1 mM IPTG at 37°C for 5 h. The cell culture was then centrifuged at 8,000 g for 5 min and the pellet was collected and kept in -20 °C until use.

2.4. Purification of TM3 and azido-TM3

The cell pellet containing FLAG-TM3 and azido-FLAG-TM3 from above was resuspended in 40 mL of ice-cold lysis buffer (20 mM Tris and 150 mM NaCl, pH 8.0) and lysed by sonication. The extract was then loaded into HisTrap FF column (GE Healthcare) charged with Ni²⁺ ions and eluted with buffer (20 mM Tris, 0.5 M NaCl and 250 mM imidazole, pH 8.0). Pooled fractions containing recombinant TM₄₅₆ were collected and then dialyzed against 20 mM Tris and 150 mM NaCl, pH8.0 to afford TM₄₅₆ or azido-TM₄₅₆ protein with LPETG motif. The FLAG tag of FLAG-TM3 or azido-FLAG-TM3 was further removed by cleavage of enterokinase, and the released TM3 or azido-TM3 was then purified by using HisTrap FF chromatography.

2.5. Incorporation of azide to TM3 via sortase-mediated ligation

TM3 (10 μM) was mixed with SrtA (5 μM) and diGly-PEG₃-azide (100 μM) in reaction buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl₂, pH 8.0). The reaction mixture was then incubated at 37°C for 2 h to obtain the C-terminal modified TM3 (azido-TM3-S). Excess diGly-PEG₃-azide was then removed by HiTrap desalting column (GE Healthcare). The obtained azido-TM3-S was further purified from the remaining reaction mixture by collecting the pass through fraction of HisTrap FF column. The total amount of pure azido-TM3-S was then measured after the purification.

2.6. Incorporation of azide to TM3 via amidation chemistry

NHS-PEG₄-azide was added to TM3 (10 μ M) in HEPES buffer pH 7.4 at a final concentration of 1 mM and the reaction mixture was incubated at room temperature for 2 h and then quenched by adding 50 mM Tris buffer for 5 min at room temperature. Then excess non-reacted NHS-PEG₄-azide was removed by using a HiTrap Desalting column (GE healthcare) to afford azido-TM3-N.

2.7. Incorporation of azide to TM3 via three-component Mannich reaction

200 μ L of 4-Azidoaniline solution (5 μ mol in 0.1 M phosphate buffer, pH 6.5), 200 μ L of formaldehyde solution (5 μ mol in 0.1 M phosphate buffer, pH 6.5), and 200 μ L of TM3 solution (5 nmol in 0.1 M phosphate buffer, pH 6.5) were added to a microcentrifuge tube and mixed well. The reaction was performed at room temperature for 48 h. The excess unreacted 4-azidoaniline and formaldehyde were then removed by HiTrap Desalting column to obtain azido-TM3-T.

2.8. Fluorescent labeling of azido-TM₄₅₆

To a solution of azido-TM₄₅₆ (10 μ M), DBCO-Cy5 was added to a final concentration of 20 μ M. The mixture was gently mixed at room temperature for 2 h in the dark. After the coupling reaction, the unreacted DBCO-Cy5 was removed by dialysis using centrifuge devices with a cutoff molecular weight of 10,000 Da. The successful ligations were then confirmed by SDS-PAGE and fluorescent images which was obtained by Typhoon 9410 fluorescence scanner (Ex/Em=633/670 nm). The in-gel fluorescence intensities were calculated and compared by Image J.

2.9. Protein C activation activity assay of TM₄₅₆ derivatives

The cofactor activities of TM₄₅₆s and azido-TM₄₅₆s were assessed by protein C activation assay as previously described with some modification [18]. TM₄₅₆ derivatives obtained from above were added into assay buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl₂, pH 8.0) containing 200 nM of human protein C (PC) to a final concentration of 50 nM, and the reaction volumes were adjusted to 100 μ L. The PC activations were initiated with the addition of human α -thrombin to a final concentration of 10 nM. After incubation for 1 h at 37°C, the PC activation was terminated by addition of 30 μ L human antithrombin III (1 mg/mL) and 2 μ L heparin (10 U/mL) for 10 min at 37°C. The activated PC was measured by incubation with chromogenic substrate H-D-Phe-Pip-Arg-pNa (0.5 mM) to determine its enzymatic activity. The increases in UV absorbance at 405 nm were measured after 20 min incubation.

2.10. Immobilization of azido-TM₄₅₆ on DBCO-coated glass

DBCO-coated glass was prepared by coating the aminosilane glass slide with DBCO-PEG₄-NHS ester (S2). For immobilization of azido-TM2 via CFCC, azido-TM2 was labeled by FITC via amine group on azido-TM2, and then 10 μ M of the FITC-labeled azido-TM2 was incubated with DBCO-coated glass slides (1.5×1.5 cm) in reaction buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) for overnight at room temperature. Next, the reaction buffer was removed from glass slide and the surface was washed with the PBS (pH 7.4) for three times to remove unreacted azido-TM2. The successful immobilization was confirmed upon examining the fluorescence image of FITC-labeled azido-TM2 (Ex/Em = 480/520 nm).

For immobilization of azido-TM3 derivatives (azido-TM3-S, azido-TM3-N and azido-TM3-T) via CFCC, anti-TM antibody was used to detect the immobilized azido-TM3. Each

azido-TM3 (10 μ M) in reaction buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) was added on the DBCO-coated glass slides (1.5×1.5 cm) and incubated for overnight at room temperature. Then the reaction buffer was removed from glass slide and the surface was washed with the PBS (pH 7.4) for three times to remove unreacted azido-TM3 derivatives. Then, mouse anti-TM antibody was used as the first antibody to capture azido-TM3 derivatives, and followed by the secondary anti-body which is FITC-labeled anti-mouse antibody. Each antibody was incubated with the slide for 1 h at room temperature, and washed three times with PBS (pH 7.4). The successful immobilization was confirmed upon examining the fluorescence image of FITC-labeled anti-mouse antibody (Ex/Em = 480/520 nm).

2.11. Protein C activation activity assay of immobilized TM₄₅₆ on DBCO-coated glass

The protein C activation activities of immobilized TM₄₅₆s (azido-TM2, azido-TM3-S, azido-TM3-N and azido-TM3-T) on glass slide were measured by protein C activation assay as previously described with some modification [18]. Assay buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl₂, pH 8.0) containing 200 nM of human protein C (PC) and 10 nM human α -thrombin were added to the surface of DBCO-coated glass slide (1.5×1.5 cm) with immobilized TM₄₅₆. After 1 h incubation at 37°C, the reaction was terminated by adding 30 μ L of human antithrombin III (1 mg/mL) and 2 μ L of heparin (10 U/mL) and incubated for 10 min at 37°C. 200 μ L of the assay buffer containing activated human protein C was transfer from the surface of DBCO-coated glass to microcentrifuge tube. The enzymatic activity of activated PC was measured by using chromogenic substrate H-D-Phe-Pip-Arg-pNa (0.5 mM), and the UV absorbance at 405 nm was measured after 20 min incubation.

3. Results

3.1. Expression and purification of recombinant TM3 and recombinant azido-TM3

In order to express protein bearing unnatural amino acid, it requires the prokaryotic expression system with auxotrophic strain. However, due to the multiple disulfide bonds in TM₄₅₆, it is challenging to express in *E. coli* because that the protein will either does not express at all or expressed as inclusion body. Previously, Chaikof *et al* [13] successfully expressed the soluble azido-TM₄₅₆ in *E. coli* B834 cell which is a methionine auxotrophic strain by fusing TM₄₅₆ to a DsbA tag in pET39b vector (TM1). Recently, we reported a recombinant TM₄₅₆ with a LPETG tag at the C-terminus used for SML [19]. After expression in *E. coli* B834 cell, we obtained the fusion protein DsbA-TM₄₅₆AL (named DsbA-TM2 in the current study) as a final product by enzymatic removal of DsbA tag. This TM₄₅₆ has the advantage to be modified by two distinguished functional groups through one-pot double modification *via* CFCC and enzymatic ligation (SML). Fusing with DsbA tag could enhance the correct folding and reduce the formation of inclusion body. However, we found that the expression yield of azido-DsbA-TM2 fusion protein is lower than native DsbA-TM2 expressed (Table 1). Further, a notable proportion of recombinant TM2 leaked into the culture medium, even at optimized expression temperature and time. Thus, only limited amount of TM2 was obtained. Moreover, DsbA tag had to be removed by thrombin cleavage to obtain the final TM2/azido-TM2, which adds extra purification steps. Therefore, we designed and expressed a new recombinant TM3 with FLAG fusion tag at the N-terminus and His-tag at the C-terminus, which can be expressed in cytoplasm to reduce the protein leaking. The designed gene fragment was inserted into pET28b (Novagen) plasmid and transformed into *E. coli* B834 cell for expression (Figure 2A and 2B). The expression yield of the native FLAG-TM3 is much higher than that of native DsbA-TM2, especially after tag removal, thus more native TM3 was obtained than native TM2. However, the expression yield of azido-TM3 was lower than azido-TM2 for unknown reasons (Table 1). There was no inclusion body observed by SDS-PAGE analysis of the

precipitate of cell culture. Therefore, we postulated that FLAG-TM3 with higher expression yield by native TM₄₅₆ expression can be used for the incorporation of azide *via* subsequent enzymatic and chemical modification.

Table 1

Figure 2

3.2. Incorporation of azide into TM3 via enzymatic method

First, we investigated an enzymatic method, sortase-mediated ligation (SML) to introduce azide group to TM₄₅₆ (TM3) at the C-terminus. Sortase A is a transpeptidase used by Gram-positive bacteria to anchor surface proteins to the cell wall through a condensation reaction between a C-terminal LPXTG motif and a N-terminal poly-(glycine) molecule [20]. The enzyme cleaves the LPXTG at the amide bond between the threonine and the glycine to form an acyl-enzyme intermediate. Subsequent nucleophilic attack by the amino group of the poly-(glycine) to the intermediate results in the formation of an LPXT-G bond and the release of the enzyme. In recent years, several groups including us reported the protein modification by using SML, such as fluorescent labeling, glycosylation, PEGylation and lipidation [19,21-24]. It has been reported that maximum reaction efficiency is generally reached by substrates with two glycines, the addition of any more glycines having no significant effect on the reaction rate [25]. Therefore, diglycine was chosen as the nucleophile in this study. By utilizing SML, azide group was introduced by a small molecule, diGly-PEG₃-azide which has a good solubility in aqueous solution and high reaction yield. With this azide introduced,

targeting functionality with larger group or poor solubility such as PEG molecule or lipid will be easily conjugated to the protein by CFCC next.

In this study, fusion TM₄₅₆ and SrtA with a His-tag at the C-terminus were prepared for easy cleanup of the unreacted TM3 and SrtA after the SML reaction. As shown in Figure 3A, due to the cleavage of T/G in LPETG motif after the SML reaction, the product azido-TM3-S did not have the His-tag, while both the unreacted TM3 and SrtA still containing the His-tag at the C-terminus could be removed from the reaction mixture by nickel affinity column so as to afford the pure azido-TM3-S efficiently. As confirmed in SDS-PAGE, the azido-TM3-S without His-tag was obtained in the flow through fraction (Figure 3B, Lane 2), and both the unreacted TM3 and SrtA were in the eluate fraction (Figure 3B, Lane 3). By comparing the amount of TM3 before SML and the pure azido-TM3-S obtained after SML, the ligation yield was around 60%. Overall, it afforded twice amount of azido-TM3-S (8.1 mg per one liter of cell culture) than direct expressed azido-TM2 (4.1 mg per one liter of cell culture) (Table 1). Next, DBCO-Cy5 was used to selectively react with azide group through CFCC confirming the successful incorporation of azide group into TM3 and its accessibility as well. As shown in figure 4A, the azido-TM3-S could be detected in fluorescent scanner as a bright red band after the fluorescent labeling. This result indicated the successful incorporation of azide functionality into a native TM₄₅₆ through SML, which could provide large amount for TM₄₅₆ modification applications.

Figure 3

3.3. Incorporation of azide into TM3 via chemical methods

Chemical modification of a native amino acid or terminals in the protein has been the main choice in the past [26]. Most modification approaches aim at a special amino acid such as Lys and Cys, or *N*-terminal amine [27]. There is no Lys in the TM3 (EGF4-6 (aa345-aa465)). Therefore, we chose *N*-terminal amine as the site-selective incorporation of azide group *via* amidation chemistry. Incubation of TM3 and NHS-PEG₄-azide at room temperature for 2 hours followed by removal of excess NHS-PEG₄-azide by desalting column afforded the desired azido-TM3-N, which was confirmed by labeling with DBCO-Cy5 as well. As a result, after the fluorescent labeling, the azido-TM3-N showed a bright red band as detected by fluorescent scanner, while non-labeled one did not show any fluorescent image (Figure 4A). In addition, in order to compare the yields of two single-site azide incorporation methods, the fluorescent intensities of DBCO-Cy5 labeled azido-TM3-S and azido-TM3-N were compared by Image J. As a result, the fluorescent intensity ratio of azido-TM3-S to azido-TM3-N is 1:1, indicating these two methods afford similar reaction yields.

Figure 4

Recently, tyrosine becomes an attractive target for protein modification because of its relatively infrequent occurrence and ability to be modified without altering protein charge [28,29]. One tyrosine-selective modification is the three-component Mannich type reaction, which uses aniline derivatives and aldehyde for highly selective tyrosine residue modification at the ortho position on phenol group, particularly it has been demonstrated that the method condition affects little if any of protein activity [28]. In our recent study, we demonstrated that the three-component Mannich type reaction occurs in mild conditions and low millimolar concentration of reagents at pH 5.5 – 6.5 and results in high yield [29]. TM3 contains three tyrosine residues and its multiple azide modification was investigated in this study. Briefly,

4-azidoaniline, formaldehyde, and TM3 were added to 0.1 M PBS buffer (pH 6.5) and incubated for 48 hours at room temperature. After desalting column, the resultant conjugate (azido-TM3-T) was obtained and was fluorescent labeled with DBCO-Cy5 through CFCC to verify the incorporation of azide as above. As a result, the azido-TM3-T showed a bright red band as detected by fluorescent scanner, while non-labeled TM3 did not show any fluorescent image (Figure 4B). Also, more than one tyrosine residue was modified on TM3 (S3). These results indicated that tyrosine-targeted methods can be used to incorporate azido functionality into the TM3 on multi-sites.

3.4. Protein C activation activity of azido-TM₄₅₆.

After successfully prepared four kinds of azido-TM₄₅₆s *via* multiple approaches above, we investigated the effect of the azide group on the activity of TM₄₅₆. TM is a potent activator of protein C. Subsequently, the activated form of protein C (APC), an anticoagulant protease, selectively inactivates coagulation factors Va and VIIIa. In this study, protein C activation activities of these azido-TM₄₅₆ derivatives were measured and compared with the unmodified TM3. The absorptions of cleaved chromogenic substrates by APC were measured at 405 nm. As a result, all the azido-TM₄₅₆ derivatives showed high APC generation activities (Figure 5). Compared with unmodified TM3, azide modified TM₄₅₆ by site-selective methods (azido-TM2, azido-TM3-S and azido-TM3-N) gave comparable activities, indicating that neither terminus nor near terminus modification of TM₄₅₆ by a single azide molecule affect the TM₄₅₆ activity. However, azido-TM3-T showed a slightly decreased activity, which may be due to the multi-site modifications on tyrosine residues of TM₄₅₆ that affect either thrombin or PC interaction related to the protein C activation.

Figure 5

3.5. Immobilization of azido-TM₄₅₆ on DBCO-coated glass

TM is mainly expressed on the surface of endothelial cell as an essential protein for the regulation of coagulation. Thus, immobilizing TM on biomaterial surface has been explored for developing antithrombotic biomaterials [30-35]. Compared with full TM, TM₄₅₆ is more suitable to prepare anticoagulant surface due to its smaller molecular weight and purer anticoagulant activity. In this study, we examined the four different azido-TM₄₅₆ derivatives obtained above for immobilization onto DBCO-coated glass so as to demonstrate their potential for biomaterial surface functionalization applications. For azido-TM2, we pre-labeled it with FITC, and then incubated with the DBCO-coated glass slide. After washing, the slide was scanned by fluorescent scanner and green spots were observed clearly (Figure 6C), while the slide with TM2 (Figure 6B) that did not contain the azide group to react with DBCO showed nothing. Similarly, azide modified TM3s (azido-TM3-S, azido-TM3-N and azido-TM3-T) were immobilized on the DBCO-coated glass slide *via* CFCC. The immobilized TM3s were later detected by anti-TM antibody and FITC-labeled anti-mouse secondary antibody. Green spots shown indicated that all three azide modified TM3s were successfully immobilized on the glass *via* CFCC reaction (Figure 6E, 6F, 6G), and no significant differences were observed among them. Further, protein C activation activities of these immobilized azido-TM₄₅₆ derivatives were measured and compared with the unmodified TM₄₅₆s (Figure 7). Consistently, TM₄₅₆ with a single azide group (azido-TM2, azido-TM3-S and azido-TM3-N) gave comparable activities, indicating that site-specific immobilization *via* either terminus or near terminus of TM₄₅₆ did not affect the TM₄₅₆ activity. However, azido-TM3-T showed a slightly decreased activity, which may be due to the random immobilization through multiple azide group conjugated to tyrosine residues of TM₄₅₆ that affect either thrombin or PC interaction related to the protein C activation.

Figure 6

Figure 7

4. Discussion

Azide is the key group in the bio-orthogonal reaction, and is often the first choice biomolecule modification *via* CFCC. Therefore, there is a growing demand of methods to introduce this small azide functionality to biomolecules easily and efficiently. Even more, to control the specific site of which amino acid is modified would benefit more, since this will maximally avoid reducing the activity of modified protein. In this study, we incorporated a single azide group to TM₄₅₆ by targeting one specific amino acid at either *N*-terminus or *C*-terminus. The most predominant way is to use methionine auxotrophic *E. coli* strain to express azido-protein by replacing the natural amino acid methionine with unnatural amino acid azidohomoalanine. However, the protein expression requires prokaryotic expression system, whereas a human protein such as TM containing multiple disulfide bonds often suffers a lower yield recombinant expression. As we found, the direct expression yield of azido-TM₄₅₆ is quite lower than native one (Table 1). Therefore, in order to increase the total amount of azido-TM₄₅₆ production, we tried to use post-expression modification to introduce azide group to TM₄₅₆ expressed with native amino acids *via* enzymatic and chemical reaction.

The enzymatic ligation we used in the study is sortase-mediated ligation in which the poly-(glycine) containing molecule is added to protein with LPEXG motif. SrtA kinetic study shows that SML requires high concentration of oligoglycine containing substrate to reach satisfying ligation efficiency. Besides, when the substrate is large or poor soluble in water,

the acyl-enzyme intermediate (LPXTG-SrtA) exists, which is hardly resolved by the oligoglycine but finally hydrolyzed by water [19,36]. This is the major problem of SML when used for larger molecules conjugation. Therefore, the substrate containing the desired functionality needs to be designed properly in order to minimize the formation of the intermediate. Pentelute *et al.* utilized SML to generate protein thioester by peptide1-LPETG and G_n-thioester, followed by native chemical ligation (NCL) to link to the peptide with N-terminal Cys [37]. This sequential ligation takes the advantage of site-specific SML using soluble and small oligoglycine substrate. Similarly, we used SML to introduce small water soluble PEG₃-azide group to TM₄₅₆, allowing the further protein modification by large, poor water soluble molecules through CFCC. Another feature is that fusion TM₄₅₆ and SrtA with a His-tag at the C-terminus facilitates a easy cleanup of the unreacted TM3 and SrtA after the SML reaction. In the reaction mixture only the azide modified TM3 does not have His-tag, thus using nickel affinity column could remove the unreacted TM3 and SrtA easily so as to afford the pure azido-TM3 efficiently (Figure 3A). Therefore, this is another advantage of using SML to introduce azide group to TM3 comparing with the other modifications resulting in a mixture of native TM3 and azido-TM3. As a result, azido-TM3-S obtained in two steps with much higher yield comparing with the direct azido-TM3 expression using azidohomoalanine (Table 1), which will facilitate site-selective modification of TM3 in large amount for practical applications.

5. Conclusions

Herein, we report a multiple strategies for site-selectively introducing azide functionality into recombinant TM₄₅₆, *via* direct recombinant expression with unnatural amino acid, chemical, and SML modification for its bio-orthogonal modification application. Overall, SML of recombinant protein affords the highest overall yield for incorporating azide functionality

into recombinant TM₄₅₆ at the C-terminus since the key protein expression step uses natural amino acids. As mentioned above, TM₄₅₆ is a small protein containing 133 amino acid while has both thrombin and protein C binding domain. Therefore, in order to retain most of its bioactivity, the best choice is to modify the *N*- or *C*- terminal of TM₄₅₆. This hypothesis was confirmed by our results that single modification maintained the protein's activity, while the multiple modifications retained less than 80% of the activity. In general, we have demonstrated a practical method for introducing azide functionality into recombinant protein for its site-selective modification application, all of which can be applied to proteins of different interests for both basic research and application.

Abbreviations:

CFCC, Copper-free click chemistry; **EGF**, Epidermal growth factor; **GG**, Glycineglycine; **PC**, Human protein C; **PEG**, Poly(ethylene) glycol; **SML**, Sortase-mediated ligation; **TM**, Thrombomodulin

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Graphical Abstract

Four different strategies to introduce azide functionality into recombinant thrombomodulin (TM), site-specifically by recombinant, chemical, and enzymatic methods at either N-terminus or C-terminus for its bio-orthogonal modification application.

Table 1. Preparation yield of pure (azido-)TM₄₅₆ per 1 L of cell culture expression

TM ₄₅₆ by initial expression	Azido-TM ₄₅₆ by post- expression modification	Final TM ₄₅₆ amount per liter expression (mg/L)
TM2		6.2
Azido-TM2		4.1
TM3		13.5
Azido-TM3		2.5
	Azido-TM3-S	8.1 ^{a)}

^{a)} Final pure azido-TM3-S amount was calculated based on the yield of SML.

Figure 1. Multiple strategies for incorporating azide functionality into recombinant TM₄₅₆ for its bio-orthogonal modification applications. (2 column)

Figure 2. Expression of TM3 and azdio-TM3. **(A):** Lane 1, purified FLAG-TM3; Lane 2, purified TM3 after cleavage and removal of FLAG tag by enterokinase and nickel affinity chromatography. **(B):** Lane 1, purified azido-FLAG-TM3; Lane 2, purified azido-TM3 after cleavage and removal of FLAG tag by enterokinase and nickel affinity chromatography. (1.5 column)

Figure 3. Preparation of azido-TM3-S by sortase-mediated ligation (SML). **(A):** Purification flowchart of SML reaction mixture. **(B):** SDS-PAGE of purification of azido-TM3-S. Lane 1, reaction mixture after desalting column; Lane 2, flow through (pure azido-TM3-S) from nickel affinity chromatography; Lane 3, eluate containing unreacted TM3 and SrtA from nickel affinity chromatography. (2 column)

Figure 4. SDS-PAGE and fluorescent image of azide modified TM3s before and after reacted with DBCO-Cy5. **(A):** SDS-PAGE and fluorescent image of azido-TM3-S (prepared by SML) and azido-TM3-N (prepared by amidation chemistry) labeled by DBCO-Cy5 *via* CFCC. **(B):** SDS-PAGE and fluorescent image of azido-TM3-T (prepared by three-component Mannich reaction) labeled by DBCO-Cy5 *via* CFCC. (2 column)

Figure 5. Protein C activation activities of TM3 and azido-TM_{456S}. Data points represent the mean $\pm$ SD, for three independent measurements. Control group contained no TM₄₅₆ at all.
(single column)

Figure 6. Immobilization of TM2, azido-TM2, TM3 and azido-TM3s onto DBCO-coated glass. (A): Schematic illustration of azido-TM3s immobilization via CFCC. (B): Control group using FITC labeled TM2 without azide for immobilization. (C): Immobilization of FITC labeled azido-TM2. (D): Control group using TM3 without azide for immobilization. (E): Immobilization of azido-TM3 obtained by SML. (F): Immobilization of azido-TM3 obtained by NHS ester reaction. (G): Immobilization of azido-TM3 obtained by three-component Mannich type reaction. For (D), (E), (F) and (G), the immobilized azido-TM3 derivatives were detected by mouse anti-TM antibody and FITC labeled anti-mouse secondary antibody. All images were obtained from Typhoon 9410 fluorescence scanner (Ex/Em=480/520 nm). (2 column)

Figure 7. APC generation activities of immobilized azido-TM2 and azido-TM3s. Tris buffer and TM3 without azide were used to incubate with the DBCO-coated glass in group “Control” and “TM3” respectively. Data points represent the mean $\pm$ SD, for three independent measurements. (single column)