SYNTHESIS AND CHARACTERIZATION OF FUNCTIONALIZED RECOMBINANT THROMBOMODULIN FOR ANTITHROMBOTIC DRUG DEVELOPMENT

LIN WANG

Bachelor of Pharmaceutical Science
Shenyang Pharmaceutical University, China
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We hereby approve this dissertation for

Lin Wang

Candidate for the Doctor of Philosophy in Clinical-Bioanalytical Chemistry Degree

for the Department of Chemistry and

CLEVELAND STATE UNIVERSITY

College of Graduate Studies

_____________________________________________________________
Dissertation Chairperson, Xue-Long Sun, Ph.D.

_____________________________________
Department & Date

_____________________________________
Anthony Berdis, Ph.D.

_____________________________________
Department & Date

_____________________________________
Baochuan Guo, Ph.D.

_____________________________________
Department & Date

_____________________________________
Nolan Holland, Ph.D.

_____________________________________
Department & Date

_____________________________________
Sihe Wang, Ph.D.

_____________________________________
Department & Date

_____________________________________
Aimin Zhou, Ph.D.

_____________________________________
Department & Date

Date of Defense: August 4th 2015
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ABSTRACT 

Thrombomodulin (TM), an endothelial cell surface membrane glycoprotein, is a cofactor for protein C activation via thrombin, and therefore a crucial regulator in protein C pathway and coagulation cascade. TM contains six epidermal growth factor (EGF)-like structures, in which the forth to sixth EGF-like region (TM$_{456}$) is the minimum functional domain responsible for protein C activation activity and could serve as a pure anticoagulant agent. However, recombinant TM$_{456}$ has a short half-life (6-9 min) that limits its therapeutic application. Modification with polymer such as PEG should be a choice to enhance the pharmacokinetics of recombinant TM$_{456}$. In addition, incorporation of protein into liposome provides several benefits including prolonged half-life and targeted drug delivery as well. Therefore, this study aimed to develop TM$_{456}$ conjugates as novel and potential antithrombotic drugs. 

Site-specific modification of protein is the key for protein conjugate preparation for either defined conjugate formation or unchanged protein activity. Firstly, a one-pot strategy for site-specific PEGylation through copper-free click chemistry (CFCC) and fluorescent labeling through sortase-mediated ligation (SML) of recombinant TM without prior chemical modification and without diminishing the protein activity has been
developed. Modification with polymer PEG aims to enhance the pharmacokinetics of recombinant TM_{456}. In addition, incorporation of a tag to the recombinant TM_{456} for subsequent detection or affinity purification facilitates efficient biological evaluation for both *in vitro* and *in vivo* experiments.

Secondly, in order to obtain the azide bearing TM_{456} for further protein modification *via* CFCC, four site-selective strategies to introduce azide functionality into TM_{456}, *via* direct recombinant expression with unnatural amino acid, chemical, and enzymatic modification were fully investigated. Among them, SML of recombinant protein affords the highest overall yield for incorporating azide functionality into recombinant TM_{456} and retained the full TM anticoagulant activity as well.

Finally, synthesis and characterization of liposome-TM_{456} conjugates were investigated by synthesizing the DSPE-PEG_{2000}-TM_{456} from azido-TM_{456} and DSPE-PEG_{2000}-DBCO *via* CFCC followed by direct liposome formation method. This liposomal formulation of TM_{456} retained the same protein C activation activity as the unmodified TM_{456}, and turned out to be much more stable in plasma than azido-TM_{456}. Further, liposome-TM_{456} showed a comparable anticoagulant effect in thrombin-induced thromboembolism mouse model by reducing the mortality from 80% to 20%. Moreover, liposome-TM_{456} enhanced the circulation time than free azido-TM_{456} *in vivo.*
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<tr>
<td>APC</td>
<td>Activated Protein C</td>
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<tr>
<td>CFCC</td>
<td>Copper-Free Click Chemistry</td>
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<tr>
<td>DBCO</td>
<td>Dibenzylcyclooctyne</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl Phosphatidyl Choline</td>
</tr>
<tr>
<td>DSPE</td>
<td>1, 2-Disteroyl-sn-Glycero-3-Phosphoethanolamine</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>NHS</td>
<td>N-Hydroxysuccinimide</td>
</tr>
<tr>
<td>PC</td>
<td>Protein C</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene Glycol</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
</tr>
<tr>
<td>rhsTM</td>
<td>Recombinant Human Soluble Thrombomodulin</td>
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<tr>
<td>SML</td>
<td>Sortase-Mediated Ligation</td>
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<tr>
<td>TCT</td>
<td>Thrombin Clotting Time</td>
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<td>TM</td>
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CHAPTER I
INTRODUCTION

1.1 Thrombomodulin and its antithrombotic activities

Cardiovascular diseases are among the most prevalent public health problems in the United States. This category includes coronary heart disease and stroke, all of which are thrombosis diseases with high mortality and morbidity. All these are considered to originate from the disfunction of platelet and coagulation. Accordingly, several antiplatelet agents such as aspirin and ticlopidine and anticoagulant agents such as heparin and warfarin were adopted and significant clinical effects were achieved during the past decades. However, several major side effects, especially bleeding, limited their clinical application. Laboratory monitoring was required for patients taking heparin and warfarin, which brings inconvenience to both patients and health care personnel and therefore very costly. In addition, these antithrombotic agents do not contribute to the prevention of restenosis after antithrombotic agents.
Recent investigations revealed the molecular bases in haemostasis which provide new target for novel antithrombotic medicine. Various antithrombosis approaches were studied, including thrombin inactivation, inhibition of thrombin synthesis, inhibition of fibrin generation, inactivation of factors involved in coagulation cascade and dysfunction of fibrin and platelet. Among these, selective inhibitors of thrombin and factor Xa, as well as members in the anticoagulant protein C system (recombinant activated human protein C or human soluble thrombomodulin) were well investigated. Some agents in this category showed promising results in Phase III clinical trial, but still had the side effects mentioned above. Due to these limitations, it is highly demanding to design novel antithrombotic agents which are with higher antithrombotic effect while less side effects and patient management requirement.

1.1.1 Structure of TM

Mature human thrombomodulin (TM) is a type I transmembrane glycoprotein with 557 amino acid residues, which is divided into five domains: N-terminal lectin-like domain (TMD1), epidermal growth factor (EGF)-like domain (TMD2), serine/threonine-rich domain containing chondroitin sulfate glycosaminoglycan (TMD3), transmembrane domain (TMD4), and cytoplasmic tail (TMD5) (Figure 1.1). Different domains are responsible for different biological functions of TM. The TMD1 domain contains 154 amino acids with a globular structure, which consists of two alpha helices, six beta strands, and a compact hydrophobic/aromatic core. TMD1 is lectin-like domain, but lacks calcium binding site in the traditional C-type lectin family. TMD1 locates in the N-terminus that far away from the plasma membrane, offering a flexible and effective
position to interact with other molecules. Studies showed that TMD1 is involved in inflammation, cell adhesion, endocytosis, and tumor growth.\textsuperscript{5-11}

\textbf{Figure 1.1} Structure of thrombomodulin (TM) with five domains. (EGF, epidermal growth factor). Reproduced from Ref. 12.
The TMD2 domain of TM is the well-characterized domain that contains six EGF-like repeats and is responsible for anticoagulation and fibrinolysis activities of TM through protein C activation and thrombin activatable fibrinolytic inhibitor (TAFI) activation, respectively. The minimum domain essential for protein C activation is the EGF456 domain, in which EGF56 is involved in the formation of thrombin-TM complex. On the other hand, the EGF3-6 repeats are essential for activation of TAFI. In addition, the TMD2 domain shows mitogenic activities on cultured Swiss 3T3 fibroblasts as well as vascular smooth muscle cells. However, it is unknown which exact EGF domain is related to these activities.

The TMD3 domain of TM is a Ser/Thr-rich domain with sites for chondroitin sulfate attachment. Studies showed that chondroitin sulfate enhances the activation of protein C via thrombin-TM complex. The enhancement of thrombin–TM binding by chondroitin sulfate may be caused by the interaction of chondroitin sulfate moiety and the anion-binding exosite of thrombin, due to the fact that heparin that binding to this thrombin exosite is an effective competitor with TM chondroitin sulfate moiety. In addition, TMD3 domain also bears N-linked and O-linked glycosylation sites. The glycosylation of this domain is not required for TM function. Studies showed that the TMD3 domain could stabilize TM in vivo because that recombinant TM with TMD1 and TMD2 domains only exhibits rapid clearance.

A highly conserved transmembrane domain of TM (TMD4) facilitates TM in the cell membrane as a type I membrane protein. The last domain of TM is the short cytoplasmic tail (TMD5), in which a cysteine is thought to be significant for the multimerization of TM. Studies confirmed that mice expressing mutant TM without
TMD5 domain show no abnormalities, which indicated that TMD5 is not essential for normal fetal development of mice, as well as survival and coagulation.\textsuperscript{24,25} However, the knockout of TM gene causes placental development defect in mice, and further leads to death of mice.\textsuperscript{26}

\subsection*{1.1.2 Biological functions of TM}

As an endothelial cell membrane glycoprotein, TM participates in many physiological and pathological processes, such as inflammation, coagulation, cancer, and embryogenesis. First, TM expressed on the endothelial cell surface acts as an anticoagulant by regulating thrombin from a procoagulant to an anticoagulant.\textsuperscript{27} Particularly, when binding to TM, thrombin is unable to activate fibrin formation or activate platelets, but is able to activate protein C in the blood. The activated protein C (APC) selectively inactivates coagulation factors Va and VIIIa as an anticoagulant protease, providing an essential feedback mechanism to prevent excessive coagulation (Figure 1.2). It has been confirmed that EGF456 in TMD2 is the minimum domain related with TM anticoagulant activity.\textsuperscript{28}

Second, TM serves as procoagulant by retarding the removal of fibrin \textit{via} TAFI activation.\textsuperscript{29} Briefly, TM activates the TAFI by thrombin, and then activated TAFI (TAFI(a) cleaves C-terminal lysine and arginine residues from fibrin. The removal of these positively charged residues suppresses the ability of fibrin to catalyze plasminogen activation and thereby delays clot lysis.\textsuperscript{30} This TM-promoted TAFI activation is opposing to TM’s anticoagulant activity and raised the question of whether this reaction might depend on different domains of TM that are required for protein C activation. Recent
study confirmed that in addition to EGF456, EGF3 of TM is also required for TAFI activation.\textsuperscript{31}

\textbf{Figure 1.2} Protein C activation-mediated anticoagulant activities of TM. TM forms a complex with thrombin, which activates protein C, and activated protein C (APC) inactivates coagulation factors Va and VIIIa with protein S (PS) and thus prevents the fibrin generation activity of thrombin. Reproduced from Ref.12.

Third, TM functions as an anti-inflammatory molecule. The TMD1 domain is involved in TM’s direct anti-inflammatory activity.\textsuperscript{6} In addition, beyond its antithrombotic property, APC, derived through TM/thrombin-mediated activation of
protein C, has anti-inflammatory activity by inhibiting monocyte/macrophage-expressing tissues factor and tumor necrosis factor (TNF)-α.\textsuperscript{32} It has been shown that APC protects baboons from Escherichia coli-induced sepsis.\textsuperscript{33} Recently, recombinant APC was proved to reduce mortality in patient with severe sepsis.\textsuperscript{34} A recombinant human APC known as Drotrecogin alfa had been approved for the treatment of severe sepsis in 2001 (Xigris, marketed by Eli Lilly and Company, Indianapolis, IN). However, Xigris was withdrawn from the market after a major study showed no efficacy for the treatment of sepsis in 2011.

Fourth, TM has antiproliferative activity. Study showed that TM inhibits smooth muscle cell proliferation,\textsuperscript{35} which could further modulate pathological alterations of the vascular wall during vein graft atherosclerosis and restenosis.\textsuperscript{36} In addition, TM has antiproliferative effect in endothelial cells.\textsuperscript{37}

Fifth, in addition to endothelial cell, TM is expressed on various tumor cells too.\textsuperscript{38} Hosaka and coworkers showed that the level of TM expression in tumor cells inversely correlates with tumor cell proliferation and invasion.\textsuperscript{38} The TM’s antiproliferative effects are due to its transmembrane and/or cytoplasmic domain, while the tumor invasion seems to be mediated through the TM’s lectin-like domain (TMD1).\textsuperscript{39}

Sixth, TM exhibits cytoprotective activity through both APC-dependent and APC-independent mechanisms. APC generated by the thrombin-TM complex induces cytoprotective effects mainly by means of binding to endothelial protein C receptor (EPCR) and activating protease receptor-1 (PAR-1) on the surface of endothelial cells and leukocytes,\textsuperscript{40} and the cytoprotection includes antiapoptotic activity,\textsuperscript{41} endothelial barrier stabilization.\textsuperscript{42} The APC-independent cytoprotection of TM involves increasing
the levels of protein myeloid cell leukemia-1 (Mcl-1), which is an antipoptotic protein via extracellular signal-regulated kinase (ERK) signaling as demonstrated with human umbilical vein endothelial cells (HUVECs) study.\textsuperscript{43}

1.1.3 Therapeutic application of recombinant TM

The most investigated recombinant TM is recombinant human soluble TM (rhs TM), which is also known as ART-123 and has been approved in Japan for the treatment of DIC, and the manufacturer used a similar genetic recombination technology for the large-scale production of the drug. The rhsTM is synthesized as a 498 amino acid, including the TMD1, TMD2, and TMD3 domains, but lacking TMD4 and TMD5 of human TM. The cDNA encoding rhsTM was initially constructed by site-directed deletion mutagenesis and expressed by CHO cells, followed by general purification to obtain the pure protein.\textsuperscript{44} The rhsTM maintains the antithrombotic effects by binding to thrombin and activating protein C as same as the native full-length TM. Overall, rhsTM has at least two forms of antithrombotic activity: (i) directly, via direct thrombin inhibition and (ii) indirectly, via APC through thrombin/TM-mediated protein C activation at the site of thrombin generation.

It was found that TM-thrombin complex is inhibited by antithrombin III (ATIII), and ART-123 is released to bind to other free thrombin and activate protein C, however, ART-123 does not affect the thrombin inactivation via ATIII.\textsuperscript{45} Studies with thromboelastography showed that ART-123 inhibits clot-induced thrombin generation, fibrinopeptide A production, and fibrin formation and thereby prevents thrombus growing.\textsuperscript{46, 47} ART-123 extends clotting time and inhibits platelet aggregation in a
concentration-dependent manner. However, ART-123 shows species specificity that it only prolonged clotting time in rat, monkey, and human plasma, but not in rabbit plasma. In the DIC models induced by tissue factor (TF) and endotoxin, a continuous infusion and a bolus injection of ART-123 blocks the hematological changes induced by TF and endotoxin, such as decrease of fibrinogen and platelets. ART-123 is effective against TF- and endotoxin-induced DIC in rats with little effect on activated partial thromboplastin time (APTT) or bleeding time, which is different from heparin that gives extended APTT. Administration of ART-123 either before or after induction of spinal cord injury (SCI) reduces compression trauma-induced SCI. ART-123 also inhibits thrombosis in a dose-dependent manner without prolonging APTT in arteriovenous (AV) shunt in rats.

Due to the advantages of ART-123 in anticoagulation, such as long half-life (around 20 hr), high bioavailability after subcutaneous injection, broad safety margin, and less bleeding in animal experiments, it has been developed to an anticoagulant. In a phase II clinical evaluation, ART-123 shows good dose-response effects for patients with DIC associated with inherent diseases, such as hematologic malignancy, solid tumor, infection. A phase III clinical study found that the resolved DIC percentage in ART-123 is 66.1%, 16.2% higher than in the heparin group. Furthermore, bleeding symptoms are significantly alleviated in ART-123 group. These indicated that ART-123 is more efficient than heparin in DIC therapy. In 2008, the rhsTM ART-123 was approved in Japan for the treatment of DIC, marketed as Recomodulin™ by Asahi Kasei Pharma, Tokyo, Japan. In October 2012, Asahi Kasei Pharma America, Waltham, MA had started
a phase III clinical trial of 800 patients for ART-123 in severe sepsis patients with coagulopathy.

In addition, ART-123 has been investigated on treatment of transplantation procedure. In the clinical study, the rhsTM successfully reduces the frequency of postoperative venous thromboembolism (VTE),\textsuperscript{56} which is a general complication in patients after elective hip replacement. Recently, the rhsTM together with ATIII concentrate has been used for two cases of sinusoidal obstructive syndrome (SOS) occurred as a complication of hematopoietic stem cell transplantation (HSCT) and the pain is effectively relieved.\textsuperscript{57} The other instance is the successful treatment of HSCT complicated by transplantation-associated microangiopathy (TAM) and sepsis using rhsTM.\textsuperscript{58} These reports suggested that ART-123 offers a promising new treatment strategy for patients with transplantation.

Another rhsTM, Solulin, consisting of TM extracellular domains with specific mutations and deletions has been investigated for further advanced purpose of resistance to proteolysis and oxidation.\textsuperscript{59} The mechanisms of Solulin such as binding active thrombin, protein C, and TAFI activation are the same as ART-123. Besides, the anticoagulant properties of Solulin are similar to ART-123.\textsuperscript{60} Study on Solulin in ischemic stroke model showed that the pretreatment with Solulin increases the time of forming stable occlusion of the middle cerebral artery and the reperfusion rate following occlusion.\textsuperscript{60} Study also showed that Solulin reduces cortical and basal ganglia infarct volumes in the case of transient middle cerebral artery occlusion.\textsuperscript{61} The underlying mechanisms of action are likely related to its anti-inflammatory effects, though they are not yet fully revealed. Recently, a phase I trial results of Solulin have been reported.\textsuperscript{62}
This is the first study of Solulin in human including the safety, tolerability, pharmacokinetics, and pharmacodynamics of Solulin in 30 healthy volunteers. The study demonstrated that Solulin is well tolerated and has good pharmacokinetics with a half-life of 15–30 hr. Therefore, Solulin shows potential for further clinical investigation as an effective and safe anticoagulant.

1.2 Bioconjugation strategies for protein functionalization

Conventional protein bioconjugation always targets functionalities on the side chains of amino acids, in which cysteine and lysine are the most frequent used ones (Figure 1.3).${}^{63}$ Sulfhydryls (-SH) in the side chain of cysteine are reactive to maleimides, haloacetyls and pyridyl disulfides, and the reactions normally take place in a neutral pH condition. Besides, cysteine is a relatively rare amino acid, leading to a more specific modification. However, some enzymes utilize cysteine as the one in active site, thereby the conjugation with cysteine definitely cause the loss of activity.
Figure 1.3 Classic bioconjugation reactions for the modification of Cys and Lys residues.

Cys residues can be modified through disulfide exchange, alkylation with iodoacetamide reagents, and Michael addition with maleimides (entries 1–3, respectively). Lys residues can be modified through amide, sulfonamide, urea, and thiourea formation with N-hydroxysuccinimide-activated esters, sulfonyl chlorides, isocyanates, and isothiocyanates (entries 4–7, respectively). Reproduced from Ref. 63.
Lysine is a basic amino acid, and in the physiological condition lysine residue contains positive charge. Thus, the primary amine on lysine residue is normally facing outside resulting in the easy accessibility for the modification. In addition, numerous amine-reactive chemical groups are developed, including N-hydroxysuccinimide esters (NHS ester), isocyanate, isothiocyanate, and sulfonyl chloride (Figure 1.3). Primary amine buffer should be avoided such as Tris (TBS), due to their competition with the primary amine on protein. But they can be used as the stop reagents. NHS ester reacts with primary amine to form a stable conjugate via amide bond. However the side reaction includes the hydrolysis of the NHS ester that will affect the final reaction yield and the hydrolysis rate rises along with the increasing of pH value.
Figure 1.4 Modern methods to modify Lys, Cys, Tyr, and Trp. Lys is modified through a reductive amination using an Ir hydride as the reductant (entry 1; bipy=bipyridyl, Cp*=${}_5\text{Me}_5$). Cys is modified through a two-step labeling procedure which involves formation of dehydroalanine and subsequent Michael addition of a thiol (entry 2), or the photochemically promoted thiol-ene reaction (entry 3; AIBN=2,2’-azobisisobutyronitrile). Tyr is modified by a nickel(II)-mediated radical coupling with magnesium monoperoxyphthalate (MMPP) as a stoichiometric oxidant (entry 4), a three component Mannich reaction with aldehyde and aniline reagents (entry 5), or a palladium-catalyzed
\( \pi \)-allylation (entry 6). Trp modification is performed using a rhodium carbenoid (entry 7). Reproduced from Ref. 63.

Besides these two residues, other amino acids can also been targeted as the modification sites, such as carboxyl group in glutamate and aspartate, phenol group in tyrosine (Figure 1.4)\(^{63}\). 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.\(^{64}\) Recently in our lab, it is 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.\(^{65}\)

**1.2.1 Click chemistry**

Sharpless and Meldal research groups developed “click chemistry”, which takes the advantage of 1,3-dipolar cycloaddition of azides with terminal alkynes to produce 1,4-disubstituted 1,2,3-triazoles. In this reaction, copper is essential as a catalyst, and therefore termed as copper catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC)\(^{66}\) (Figure 1.5). Different than the 1,3-dipolar cycloadditions the Rolf Huisgen introduced which requires in harsh condition, CuAAC can be performed in aqueous solution and showed up a 7-fold enhanced reaction efficiency than the uncatalyzed one.\(^{67}\) CuAAC also showed high specificity and selectivity because of the inert property of azide and alkyne groups. All these features make this reaction gain wide interest in a lot of areas, including polymer chemistry, materials chemistry and chemical biology.\(^{63, 68-71}\) However, one significant drawback of this reaction is the application in living system due to the toxicity
of copper(I). Bacteria have been found to stop dividing after the labeling via CuAAC, even though they can survive in the reaction. It has been also reported that CuAAC can cause neurological disorders, hepatitis or kidney diseases in vivo.

Figure 1.5 Bioorthogonal [3+2] cycloadditions of azides and alkynes to form triazoles. Terminal alkynes are activated by Cu to undergo cycloaddition with azides under physiological conditions (top). Cyclooctynes react with azides through a strain-promoted [3+2] cycloaddition (bottom). Reproduce from Ref.63.

In order to improve the biocompatibility of click chemistry, it has been tried find a way that can avoid using of copper. It turned out that the cyclooctyne bearing different functionalities can efficiently react with azide, and this reaction is named as strain-
promoted azide–alkyne cycloaddition (SPAAC) (Figure 1.5). Since this kind of reaction is in the absence of copper, it is also called copper-free click chemistry (CFCC). The first generation of SPAAC is slower than CuACC or Staudinger ligation, however, the Bertozzi group discovered that with the help of two fluorine atoms (difluorinated cyclooctyne, DIFO), the reaction rate increases more than 50 times compared to the parent compound. Around the same time, the Boons group developed the strain-promoted cycloaddition by using dibenzocyclooctyne (DBCO), which achieves a similar reaction rate as DIFO without toxicity. Therefore, strain-promoted cycloaddition is employed to solve many problems in chemical biology, pharmaceutical science and materials science.

1.2.2 Sortase-mediated ligation

Sortases are membrane protein in Gram-positive bacteria, responsible for the catalysis of anchoring surface protein to the bacterial cell wall, which is a significant step involving in both physiology and pathogenesis. Sortase is a superfamily, and many Gram-positive bacteria contain more than one sortases in the genome. For instance, srtA and srtB genes encoding the corresponding enzymes both exist in Staphylococcus aureus, in which sortase A (SrtA) is the “housekeeping sortase” that can recognize LPXTG motif in the protein. After Schneewind and co-workers first discovered SrtA in S. aureus [37], researches are focused on this transpeptidation reaction and its application in protein modification.
Figure 1.6 Principles of sortase-mediated ligation (SML). Sortase A first recognizes an LPXTG sequence within polypeptide 1 and cleaves the amide bond between the Thr and the Gly with an active-site Cys184, generating a covalent acyl-enzyme intermediate. The thioester intermediate is then attacked by an amino group of the oligoGly-containing polypeptide 2, which allows the ligation of the two polypeptides by a native peptide bond. Reproduced from Ref. 82.

Wild-type SrtA is a type II membrane protein consisted with 206 amino acids with an N-terminus transmembrane domain. In order to easily express and purify the recombinant SrtA, it is engineered by the removal of the transmembrane domain (normally the first 59 amino acids at N-terminus) and addition of His-tag. This truncated
soluble SrtA can be expressed by *E. coli* with high yield and is widely used by scientists.\(^{83,84}\) Sortase A can recognize a C-terminal LPXTG motif in the protein, and the active-site cysteine (Cys184) attacks the carbonyl carbon between Thr and Gly to form LPXT-SrtA, a covalent acyl-enzyme intermediate. Subsequent nucleophilic attack by the amino group of the poly-(glycine) to the intermediate results in the formation of an LPXT-GGG bond and the release of the enzyme (Figure 1.6). If there are no oligoglycine nucleophiles, the acyl-intermediate is hydrolyzed by water.\(^{82}\) SrtA is extremely specific for the substrates containing LPXTG motif and N-terminal Gly repeats, and only when both are satisfied, the proper final product will be obtained.\(^{85,86}\) X represents all natural amino acids except Cys and Trp, meaning all the other 19 amino acids don’t affect the recognition at X position for SrtA.\(^{85}\) The native substrate containing oligoglycine for SrtA is lipid II with pentaglycine, but any substrate with tryglycine or diglycine shows similar reaction efficiency.\(^{86,87}\)

The highly specificity and the mild reaction condition make SrtA an excellent “stapler” for two molecules, and the reaction is known as “sortase-mediated ligation” (SML). SrtA has been used for protein conjugation to introduce new functionalities to proteins and even protein-protein conjugation, immobilization of protein to solid surface. It also can be applied for live cells,\(^{82}\) for example, the first try is that by Ploegh and coworkers used SrtA to label MHC H-2Kb protein on live cells by different oligoglycine probes.\(^{88}\)

### 1.3 Liposomes as drug delivery systems

Liposomes are self-assembling lipid bilayers that comprises of an outer lipid bilayer enclosing an inner aqueous compartment.\(^{89}\) Over the past few decades, liposomes have
been extensively studied as a carrier system for therapeutically active compounds because of their special characteristics. First of all, liposomes can encapsulate hydrophilic and hydrophobic and even amphipathic drugs. Hydrophobic drugs are predominantly entrapped in the lipid bilayer of liposomes away from the aqueous environment due to their bad water solubility. While there are two locations for hydrophilic drugs, either the aqueous core of the liposome or the external aqueous solution. Secondary, liposomes have excellent biocompatibility, low toxicity and no activation on immune system. Third, liposomes can protect drugs from the external environment and increase the stability of drugs.

Liposomes are classified into different types according to the size range, preparation methods, or composition. For example, based on size range, liposomes are classified into two categories, multilamellar vesicles and unilamellar vesicles; Unilamellar vesicles consist of single bilayer and including three classes, small unilamellar vesicles (SUV) whose size ranges 20-40 nm, medium unilamellar vesicles (MUV) whose size ranges 40-80 nm and large unilamellar vesicles (LUV) whose size ranges 100-1000 nm. Based on the composition, there are three classes, including conventional liposome, stealth liposome and targeted liposome (Figure 1.7).

1.3.1 Conventional liposomes

Conventional liposomes are known as the first generation of liposome as drug carrier, and the major components of this kind of liposome are natural phospholipids or their derivatives such as 1,2-distearoryl-sn-glycero-3-phosphatidyl choline (DSPC) and egg phosphatidylcholine. By using this formulation, there are two advantages comparing with the free drugs. One thing is this bilayer can protect the encalsulated drugs.
from \textit{in vivo} degradation; the other thing is liposomes can passively target some tissues or organs such as the liver, spleen, because the large molecules such as liposomes are easily enter these tissues or organs with discontinuous endothelium. \footnote{92}

However, such a liposomal formulation has some drawbacks and the most important one is the instability in plasma. \footnote{97-99} For particles smaller than 8 nm, they are primarily excreted \textit{via} urine from body; for particles larger than 8 nm, such as liposome, a main clearance pathway is by the mononuclear phagocyte system (MPS) or reticuloendothelial system (RES) which is a part of immune system and composed of phagocytic cell. This feature benefits the targeted drug delivery when the drugs are used for treating infections inside in the MPS. While, once the target site is out of this system, liposomes are quickly removed from blood by RES. The liposome clearance by MPS has been proved to be related with the size of liposome. \footnote{95} Therefore, control the size of the liposome can also minimize the uptake rate of liposome by MPS. Large size liposomes of 500-5000 nm and small size liposomes of 20-50 nm both have a relatively shorter half-life comparing with liposomes of 50-200 nm. \footnote{95, 97, 100} Moreover, in order to stabilize the liposome and reduce the drug releasing rate, cholesterol can be incorporated into the liposome. Cholesterol can alter the fluidity of the lipid bilayer, and make it more rigid, resulting in a relatively slow drug release. \footnote{101} Even though only by the addition of cholesterol or size control cannot fully overcome the challenges of conventional liposomes, such as short half-life, several drugs including Ambisone, Myocet, Daunoxome and Daunorubicin using this kind of liposome have been clinically approved (Table I). \footnote{102-104}
1.3.2 Stealth liposomes

As mentioned above, liposomes and particles larger than 8 nm are mainly cleared by MPS from the blood circulation. The uptake of liposomes by MPS is mediated by serum proteins, opsonins that can bind to the surface of liposome and enhance the detection of liposomes by MPS. Based on these observations, a new generation of liposomes containing the groups can shield liposome from opsonization, and named stealth liposome was developed. By modifying the surface of liposome with a hydrophilic polymer or a glycolipid containing flexible chain can occupy the space around the liposome, therefore block the binding between of liposome and biomolecules in blood, especially opsonins by the steric effect.

There are many kinds of polymers developed used to prepare stealth liposome, among which poly-(ethylene glycol) (PEG) is the most popular one as polymeric steric stabilizer. PEG is a linear polyether diol with good biocompatibility and solubility in aqueous solution. It also has many other properties that suitable for its application in biology system, such as the very weak immunogenicity and antigenicity. PEG is on the FDA’s compounds Generally Recognized as Safe (GRAS) list and safe for internal consumption, including foods and pharmaceuticals.

PEG molecule can be incorporated on the liposomal surface by diverse methods, and one of them is to anchor the polymer by lipid bearing a cross-linked functionality, such as PEG-distearoylphosphatidylethanolamine (DSPE). The density and length of PEG chain grafting on liposome surface has also been studied, and it indicates that 2 mol% DSPE-PEG2000 showed the longest plasma circulation lifetimes, even though the increasing of half-life was observed as low as 0.5 mol% of DSPE-PEG2000.
After anchoring PEG on liposome surface, many new features could be obtained for this stealth liposome. This kind of liposome intensely decreases MPS uptake, and therefore enhances pharmacokinetic properties. The prolonged blood circulation time will further facilitate the biodistribution in perfused tissues. In addition, the cover by PEG chain reduces the surface-surface interaction of liposomes to prevent aggregation, and liposomes become more stable. Successful examples of PEGylated liposome include PEGylated liposomal vincristine (Marqibo, Talon Therapeutics, Inc.) (Table I). Vincristine is one of the most widely used and more effective drugs in paediatric oncology, after using PEGylated liposomal formulation, it shows a 40- to 66-fold increasing of half-life compared to free vincristine.\textsuperscript{107, 108} Another exceptional example is DOXIL/Caelyx which is used for the treating many kinds of cancers including leukemia, breast cancer, bone cancer and so on. DOXIL is the PEGylated liposomal formulation of doxorubicin whose circulation time is extended by 100 times than free doxorubicin and is the first FDA approved nano-drug.\textsuperscript{109}

1.3.3 Targeted liposomes

Even though stealth liposome solves a major problem in blood circulation in the liposome application, it is still not perfect since it undergoes the passive targeting that might lead to suboptimal pharmacokinetics.\textsuperscript{110} Therefore, the targeted liposome has been developed to fulfill this requirement. A best way to achieve the targeted liposome is to conjugate the targeting moiety on the liposome surface, and varied types of molecule which have the affinity with the targeted cells or tissues can be used including antibodies,
peptide, glycoprotein and carbohydrate.\textsuperscript{111-113} In addition, the targeting moiety can combine with stealth liposome by conjugating to PEG molecule to obtain bifunctional liposome with long half-life and targeted delivery. Targeted liposome increases markedly the local drug concentration in the targeted tissue or cells without harmful side effect on healthy cells.
Figure 1.7 Structural and design considerations for liposomal drug delivery. Liposomes can be surface functionalized to obtain stealth through PEGylation and to promote receptor-mediated endocytosis by using targeting ligands such as antibodies, proteins, carbohydrates, and so on. Polyethylene glycol (PEG) density determines its structure at the liposome surface, with densities below 9% adopting a mushroom-like globular structure and those above 9% adopting a more rigid, extended, brush-like morphology. Chemotherapeutics or diagnostics can be encapsulated into the aqueous lumen, incorporated into the lipid bilayer, or conjugated to the liposome surface. Reproduced from Ref. 110.
1.3.4 Preparation methods of liposomes with ligand

1.3.4.1 Direct liposome formation with ligands

For most small ligands, the common method for the liposome surface modification is direct liposome formation. In this strategy, the key lipid-ligand conjugate will be firstly synthesized, and then the liposome consisting of all lipid components will be formulated. Using this method, it is inevitable that about half of total ligands is facing the inside of liposome and therefore will not have the capability to interact with their target molecules. For ligands that are inexpensive and of large quantity, such as saccharides and vitamins, it is an appropriate method.\textsuperscript{114}

1.3.4.2 Post-insertion approach

In order to solve the problem in direct liposome formation as mentioned above, another method was invented. In this method, liposome was first formed, followed by the incorporation of functionalized ligands and lipid tailor. The ligands were linked to lipid with covalent bond and micelles were formed in aqueous solution. The micelles with ligands will be then mixed with liposome so that ligands can fuse into liposome. Lipid-ligand conjugates in solvent are usually low in solubility and stability or are not compatible with various steps of manufacture, such as high temperature. This method liposome surface modification requires less ligands and results in noncovalent and reversible binding, therefore is superb for antibody protein or peptides. However, this method has its own disadvantages, including low insertion rate and low solubility and stability of lipid-ligand conjugates in solvent. Due to these, the applications of this method are limited.
1.3.4.3 Post-functionalization approach

Post-functionalization is a chemical modification approach in which biomolecules were incorporated onto the surface of performed vesicles containing functionalized (phospho) lipid anchors. Till now, several approaches have been tried successfully, such as amide or thiol-maleimide coupling or by imine or hydrazone linkage.\textsuperscript{115}

**Table I. Benefits of drug load in liposomes**

<table>
<thead>
<tr>
<th>Benefits of drug load in liposome</th>
<th>Examples</th>
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</thead>
<tbody>
<tr>
<td>1. Improved solubility of lipophilic and amphiphilic drugs</td>
<td>Amphotericin B, porphyrins, minoxidil, some peptides, and anthracyclines, respectively; hydrophilic drugs, such as anticancer agent doxorubicin or acyclovir</td>
</tr>
<tr>
<td>2. Passive targeting to the cells of the immune system, especially cells of the mononuclear phagocytic system</td>
<td>Antimonials, amphotericin B, porphyrins, vaccines, immunomodulators</td>
</tr>
<tr>
<td>3. Sustained release system of systemically or locally administered liposomes</td>
<td>Doxorubicin, cytosine arabinoside, cortisones, biological proteins or peptides such as vasopressin</td>
</tr>
<tr>
<td>4. Site-avoidance mechanism</td>
<td>Doxorubicin and amphotericin B</td>
</tr>
<tr>
<td>5. Site-specific targeting</td>
<td>Anti-inflammatory drugs, anti-cancer, anti-infection</td>
</tr>
<tr>
<td>6. Improved transfer of hydrophilic, charged molecules</td>
<td>Antibiotics, chelators, plasmids, and genes</td>
</tr>
<tr>
<td>7. Improved penetration into tissues</td>
<td>Corticosteroids, anesthetics, and insulin</td>
</tr>
</tbody>
</table>

Reproduced from Ref.116.
1.4 Research Design and Rational

New mechanisms have been investigated for antithrombotic drug development based on haemostasis. Particularly, endothelial thrombomodulin (TM) has the ability to alter thrombin’s procoagulant activities to anticoagulant activities. Even more, the complex of thrombin and TM will activate protein C and subsequently the activated protein C will further has the anticoagulant activities. In some conditions such as inflammatory stimuli, direct vessel wall injury and oxidant stress, TM expression is down regulated, which then increases the risk of thrombosis formation. Therefore, TM will be more aimed at these kinds of thrombosis related diseases. However, TM also has other biological functions including procoagulant activity via TAFI activation by domain EGF3-6. While the minimum domain for protein C binding and activating is EGF456. Thus, only domain EGF456 (TM$_{456}$) is a pure anticoagulant candidate. However, the major disadvantage of this protein is the short half-life, which is only 6-9 min as determined in rats and monkey. This in fact markedly limits the application of TM$_{456}$ as antithrombotic agent.

Protein modification by special functionalities such as PEG can improve the pharmacological properties for protein drug. Further, liposomes have been extensively studied as cell surface model as well as carrier for delivering small drugs, proteins and genes to the body. Particularly, incorporation of protein drug with stealth liposome always enhances the blood circulation time. Besides, the liposomal formulation of TM$_{456}$ could mimic the membrane protein nature of full TM$_{456}$, and help it to achieve better activity. Therefore, we hypothesized that the incorporation of TM$_{456}$ with PEG or liposome provides a strategy for generating novel and potential antithrombotic drug.
On the other hand, copper-free click chemistry has been proven to be a powerful bioconjugation tool for protein in mild condition with high efficiency, and is suitable for TM_{456} modification by PEG molecule or liposome. Moreover, in order to retain TM_{456}'s anticoagulant activity, site-specific modification should be performed, since TM_{456} is a small size protein but bearing two binding sites for thrombin and protein C, and multi-site modification is easy to disturb these binding sites. Therefore, we found out a way that afforded high yield of azido-TM_{456} with single site modification which then can be used for further conjugate with PEG or lipid via copper-free click chemistry to obtain a pharmacokinetic properties enhanced TM_{456} conjugate.

1.5 References


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CHAPTER II
CHEMOENZYMATIC BIO-ORTHOGONAL CHEMISTRY FOR SITE-SPECIFIC DOUBLE MODIFICATION OF RECOMBINANT TM456AL

2.1 Introduction

Protein modification is extremely useful for both understanding of protein structure and function and the mechanism of the biological pathways that the protein is involved. For example, protein labeling can facilitate protein localization, binding partner identification, and purification both under native or denaturing conditions.\textsuperscript{1} Furthermore, modification of protein can expand the proteins’ functional capacity, especially, for therapeutic proteins, which is very essential for enhancing their pharmacodynamic and pharmacokinetic properties.\textsuperscript{2} The key point for a practical protein modification is to carry out site-specific chemistry so as to avoid upsetting the protein activity due to random modification since there is a diversity of chemically reactive functionalities in the protein
in general. Particularly, site-specific attachment of two or more different functionalities to a protein is even more challenging than introducing a single modification but is highly sought after.\(^3\) For example, introducing an analytical probe at one site for protein tracking and a functional moiety at the other site for expanding its functional capacity offers an enormous way to generate therapeutic proteins with enhanced biological activity and stability while affording efficient biological activity evaluation both \textit{in vitro} and \textit{in vivo} as well.

Bio-orthogonal chemistry has been developed for selective and efficient modification of proteins.\(^4\) The most widely used approaches are azide-reactive Staudinger ligation, copper(I)-catalyzed click chemistry and Copper-free click chemistry (CFCC).\(^5\) These approaches benefit from the azide functionality since it is small, bears no overall charge and can be introduced with relative ease to a wide variety of structures, often without disruption to biological behavior of proteins. On the other hand, enzymatic ligation has been explored for selective protein modification in native condition as well.\(^6\) For example, sortase-mediated ligation (SML) has been utilized to site-specifically modify proteins.\(^7\) Specifically, SrtA recognizes a unique pentapeptide LPXTG, where \(X\) is variable, of the C-terminal domain of target proteins and transfers the carboxylic group of Thr to a substrate carrying an \(N\)-terminal glycine so to afford the transpeptidation products. Herein, we envision that the combination of CFCC and SML will provide a practical strategy for site-specific double modification of proteins with specific purpose, such as site-specific poly(ethylene glycol) (PEG) modification and fluorescent labeling (Figure 2.1).
Thrombomodulin (TM), a membrane glycoprotein predominantly expressed by vascular endothelial cells, is involved in many biological processes such as thrombosis and inflammation.\(^8\) TM is a cofactor for protein C activation with thrombin, and thus a crucial regulator in protein C pathway and coagulation cascade.\(^9,10\) Recombinant TM\(_{456}\) has shown promising antithrombotic activity, however, the short half-life (6-9 min) limits its therapeutic application as an anticoagulant agent.\(^11\) Modification with polymer such as PEG should be a choice to enhance the pharmacokinetics of recombinant TM\(_{456}\).\(^12\) In addition, incorporation of a tag to the recombinant TM\(_{456}\) for subsequent detection or affinity purification will facilitate efficient biological evaluation for both *in vitro* and *in*
vivo experiments. Herein, I report a straightforward and robust site-specific double modification of recombinant TM456, namely, PEGylation via CFCC and tagging with a variety of functionalities such as fluorescent dyes or affinity handles via SML concurrently.

2.2 Results and discussion

2.2.1 Expression and purification of TM456AL

As mentioned above, all three EGF domains of TM456 are critical for the interaction of TM with thrombin and protein C. Thus, I proposed a site-specific modification at the C-terminal of TM456 without diminishing its activity. In previous study, I expressed a TM456 derivative with C-terminal LPETG tag for its end-point immobilization via SML, where the activity of immobilized TM456 was successfully retained. In the present study, I designed a recombinant TM456 derivative with azidohomoalanine for CFCC modification and LPETG tag for the recognition of SrtA both at the C-terminal of TM456 (TM456-Azide-LPETG, named as TM456AL). In addition, since TM456 contains 9 pairs of disulfide bonds, for the proper folding of recombinant protein, a DsbA together with a His tag were fused to the N-terminal of TM456 protein (DsbA-His6-TM456AL), which was expressed in E. coli. B834. The targeted TM456AL was obtained after enzymatic digestion of DsbA-His6-TM456AL, followed by removal of DsbA-His6 with Nickel affinity column and further purification with HiTrap Q chromatography. The resultant TM456AL was analyzed by SDS-PAGE (Figure 2.2A).
2.2.2 Double-labeling of TM2 with DIBO-AF647 or/and Gly2-Bodipy

With the purified TM456AL in hand, we first examined site-specific fluorescent labeling via CFCC and SML, respectively. Two fluorescent probes with different wavelengths were used in order to selectively confirm the successful labeling. A commercial DIBO-Alexa Fluor 647 (DIBO-AF647) (Invitrogen) was used for CFCC labeling, while a synthesized Gly2-Bodipy was used for SML labeling of TM456AL. As shown in the SDS-PAGE gel resulting from Coomassie blue staining and fluorescent imaging, the two fluorescent probes were successfully conjugated onto the TM456AL via CFCC and SML, respectively (Figure 2.2B, lane 2 and 3). Next, double labeling of TM456AL was performed and the fluorescent intensity for both ligations were observed and appeared comparable (Figure 2.2B, lane 4). These results indicated that the one-pot CFCC and SML reaction can be applied for site-specific double labeling of the TM456AL.
Figure 2.2 SDS-PAGE analysis and in-gel fluorescence analysis of the double labeling reactions. (A) SDS-PAGE analysis of purified recombinant TM proteins: Lane 1. DsbA-His\textsubscript{6}-TM\textsubscript{456}AL fusion protein, lane 2. Thrombin treated DsbA-His\textsubscript{6}-TM\textsubscript{456}AL, lane 3. Purified TM\textsubscript{456}AL from HiTrap Q column eluate; (B) SDS-PAGE analysis and in-gel fluorescence analysis of the double labeling reactions of TM\textsubscript{456}AL (10 µM) with DIBO-AF647 (10 µM) and/or Gly\textsubscript{2}-Bodipy (200 µM) in the presence of SrtA (2 µM) in 20 mM HEPEs (pH 7.4), 150 mM NaCl and 5 mM CaCl\textsubscript{2} at 37 °C for 1 h.

2.2.3 Optimization of one-pot double-modification reaction conditions

Studies show that CFCC is very efficient in protein modification,\textsuperscript{14} while SML is a reversible reaction\textsuperscript{15} and thus requires further optimization to increase the overall efficiency of the proposed double-modification. A previous kinetic study of SML
suggested some solutions to enhance its efficiency, such as using high concentration of LPXTG tagged substrate and excess of oligo-glycine nucleophile, as well as removal of product during reaction.\textsuperscript{16} Therefore, we evaluated the reaction conditions that satisfy both ligation methods in the one-pot reaction of CFCC and SML. First, a stable TM\textsubscript{456AL} concentration of 10 µM was optimized for all its modifications below since higher concentrations induce precipitations in the reaction solutions, which is most likely due to its rich cysteine residues that form multiple intermolecular disulfide bonds easily at high concentration. Then, the molar ratio between TM\textsubscript{456AL} and reactant dyes was investigated for both CFCC and SML labeling (Figure 2.3A). As summarized in Table II, 5 equivalents of DIBO-AF647 were required for a sufficient CFCC-mediated labeling, while at least 20 equivalents of Gly\textsubscript{2}-Bodipy were required for SML to obtain a satisfactory labeling efficiency. In addition, the pH value was investigated for both reactions. As a result, pH value does not affect the CFCC labeling, while slightly basic conditions (pH 7-9) benefit the SML reaction (Figure 2.3B).

By using the reaction conditions optimized above, reaction kinetics of both SML and CFCC were investigated. Based on the fluorescence intensities of one-pot reaction products (Figure 2.4), CFCC-mediated labeling reached an approximate maximum in 2 hours, whereas, it took 3 hours for SML. It must be noted that the reaction time should not last indefinitely since SrtA irreversibly hydrolyzes the LPXTG motifs of both diglycine substrate and product as previously reported.\textsuperscript{15} Taken together, an optimized reaction condition, 50 µM alkyne reactant, 400 µM diglycine nucleophiles and 5 µM SrtA in Tris buffer pH 8.0 with 5 mM Ca\textsuperscript{2+} for 3 hours, was used in the following one-pot double modification of TM\textsubscript{456AL}.
Figure 2.3 Optimization of one-pot double-modification reaction conditions. (A) Optimizing dyes (DIBO-AF647 and Gly2-Bodipy) concentrations by using ratio of dye to TM_{456}AL from 1:1 to 1:200; (B) optimizing reaction pH from 3.0 to 9.0; (C) optimizing of SrtA to TM_{456}AL ratio from 0.001 to 1.1; (D) optimizing reaction temperatures from 4°C to 37°C. In-gel fluorescence intensities calculated and compared by Image J.
**Figure 2.4**  Optimization of reaction time of one-pot double labeling of TM$_{456}$AL via SML and CFCC: TM$_{456}$AL (10 µM) was incubated with Gly$_2$-Bodipy (400 µM), SrtA (5 µM) and DIBO-AF647 (50 µM) in 20 mM Tris (pH 8.0), 150 mM NaCl and 5 mM CaCl$_2$ at 37 °C for different time (1-240 min): (A) SDS-PAGE followed by fluorescent scanning (Bodipy: Ex/Em=488/526 nm; AF647: Ex/Em = 633/670 nm) and (B) in-gel fluorescence intensities calculated and compared by Image J. The highest intensity of SML or CFCC was set as 100%. Data points represent the mean ± SD from three independent experiments.
Table II. Optimized one-pot double modification reaction conditions

<table>
<thead>
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<th>Factors</th>
<th>SML</th>
<th>CFCC</th>
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<tbody>
<tr>
<td>Gly2-R1 : TM456AL (molar)</td>
<td>≥ 20:1</td>
<td>NA</td>
</tr>
<tr>
<td>Alkyne-R2 : TM456AL (molar)</td>
<td>NA</td>
<td>≥5:1</td>
</tr>
<tr>
<td>SrtA : TM456AL (molar)</td>
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<td>NA</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>7.0 to 9.0</td>
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<tr>
<td>Reaction time</td>
<td></td>
<td>≥ 3 hours</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td>RT to 37 °C</td>
</tr>
</tbody>
</table>

2.2.4 One-pot PEGylation and fluorescent labeling of TM456AL

As mentioned above, PEGylation is a common method to stabilize protein and fluorescent dye labeling facilitates protein tracking in biological system. In the present study, we tested the feasibility of one-pot PEGylation and fluorescent labeling of TM456AL via CFCC and SML. The first combination was CFCC-mediated PEGylation with DBCO-PEG5000-OMe (Click-Chemistry Tools) and SrtA-mediated fluorescent labeling with Gly2-Bodipy. As shown in SDS-PAGE and western blot by using anti-human TM mAb (Figure 2.5A and B), double modification of TM456AL with PEG and Bodipy afforded a new band with a 7 kDa molecular weight increase, which corresponds
to the molecular weight of DBCO-PEG$_{5000}$-OMe. More than 90% TM$_{456}$AL was PEGylated within 3 hours compared to the unreacted TM band in the control experiment, proving an excellent yield for CFCC in one-pot reaction. Furthermore, a monoclonal Ab E11 (Academia Sinica), which specifically recognizes the backbone of PEG molecule, was used in western blot to confirm the PEG conjugates (Figure 2.5D) and fluorescent imaging was carried out to confirm Gly$_2$-Bodipy anchored onto TM$_{456}$ (Figure 2.5C), respectively. Moreover, the double modified product (Figure 2.5, lane 4) exhibited comparable signal strength to the individual reactions (Figure 2.5, lane 2 and 3), demonstrating that the SML and CFCC could take place in one-pot reaction without interfering with each other.
Figure 2.5 One-pot PEGylation via CFCC and fluorescent labeling via SML of TM_{456}AL: (A) SDS-PAGE Coomassie blue staining, (B) western blot analysis using anti-human TM mAb, (C) fluorescent image (Ex/Em = 488/526 nm), and (D) western blot analysis using anti-PEG mAb E11 of. The reactions of TM_{456}AL (10 μM), SrtA (5 μM), Gly2-Bodipy (400 μM) and DBCO-PEG5000-OMe took place in 20 mM Tris (pH 8.0), 150 mM NaCl and 5 mM CaCl$_2$ at 37°C for 3 h.

2.2.5 PEGylation of TM_{456}AL via sortase-mediated ligation with Gly2-PEG5000-OMe

Alternatively, the opposite set of the double modification was examined, in which PEGylation with Gly2-PEG5000-OMe via SML and fluorescent labeling with DIBO-AF647 via CFCC were performed. The PEGylated product around 35 kDa was confirmed by western blot using anti-PEG mAb (Figure 2.6A and B), the molecular weight being
similar to that observed for PEGylation \textit{via} CFCC. However, this PEGylation product (Figure 2.6A, lane4) presented in low yield (less than 10%), when compared to control sample (Figure 2.6A, lane2). In addition, a 40 kDa product was observed in a considerable amount, which was identified as the complex of TM$_{456}$AL and SrtA (Figure 2.6A, lane 3 and 4). In SML, Cys184 first reacts with LPXTG tagged substrate to form LPXT-SrtA, a covalent acyl-enzyme intermediate, which is subsequently approached by the oligo-glycine nucleophile to form the conjugate of two substrates.\cite{17} Our result indicated that the 40 kDa product was the acyl-SrtA intermediate and barely reacted with the second substrate Gly$_2$-PEG$_{5000}$-OMe, which was probably due to the steric issues resulting from the size of the target substrate. To confirm this speculation, SrtA-mediated modification of TM$_{456}$AL with molecules of different sizes containing N-terminal diglycines were tested. Herein, FITC-labeled SrtA was used for easy confirmation of the intermediates in SDS-PAGE. As a result, large quantities of intermediates formed in the absence of oligo-glycine substrate (Figure 2.6C, lane 2), as well as in the presence of Gly$_2$-PEG$_{5000}$-OMe (Figure 2.6C, lane 6). In contrast, in the group of small nucleophiles, such as pentaglycine, Gly$_2$-Biotin and Gly$_2$-Dansyl, there was significantly less intermediates observed in the fluorescent image (Figure 2.6C, lane 3-5) but more conjugates formed (Figure 2.7). These results indicated that small nucleophilic substrate resolves the acyl-enzyme intermediate more efficiently than large size substrates such as PEGs due to the steric issues.\cite{18} Therefore, to cost effectively produce double-modified conjugates, we utilized CFCC for PEGylation and SML for linking of diverse small molecules in larger scale reactions.
Figure 2.6 PEGylation of TM_{456}AL via SML with Gly_{2}-PEG_{5000}-OMe: (A) SDS-PAGE analysis of reactions between SrtA (5 µM), TM_{456}AL (10 µM) and Gly_{2}-PEG_{5000}-OMe (400 µM); (B) western blot analysis using anti-PEG mAb E11; (C) in-gel fluorescent image of reactions between FITC-labeled SrtA ((Ex/Em = 488/526 nm, 5 µM), TM_{456}AL (10 µM) and diglycine nucleophiles (400 µM). All reactions were performed in 20 mM Tris (pH 8.0), 150 mM NaCl and 5 mM CaCl$_2$ at 37 °C for 3 h.
Figure 2.7 Preparation of double modified TM456AL via one-pot reactions by using CFCC for PEGylation and SML for other bioanalytical probes. (A) SDS-PAGE of the double modified TM456AL; (B) Confirmation of the double modified TM456AL with Bodipy and Biotin by fluorescent scanning and the one with Dansyl under UV.

2.2.6 Protein C activation activity of TM456AL and TM456AL conjugates

After confirming the proper ligation strategy, we further tested the influence of the double-modification on the activity of TM456AL modified with DBCO-PEG5000-OME via CFCC and three diglycine functionalities (Bodipy, Biotin and Dansyl) via SML. In this study, protein C (PC) activation activities of these conjugates were measured and compared with the unmodified TM456AL. The absorptions of activated protein C (APC) cleaved chromogenic substrates were measured at 405 nm and then compared. The rate of activating PC by thrombin is quite slow in the absence of TM456AL (Figure 2.8, column 1), while in the presence of TM456AL and those double-modified conjugates, the
generation of APC was significantly enhanced (Figure 2.8, column 2-6). There was no significant change in PC activation activities of dual-modified conjugates by compared to unmodified TM, indicating that the one-pot double modification does not hamper the TM activity. It has been reported that the activity decline of immobilized TM may be caused by random modification, whereas in our previous work, the activity of TM was fully retained after the site-specific functionalization and immobilization of TM via SML. Our study here further demonstrated that it is feasible to immobilize TM and introduce other functionalities at the same time without affecting its activity, which will be used in anticoagulant materials with multi-functions in the future.

**Figure 2.8** Activated protein C (APC) generation by TM and its derivatives via CFCC and SML modification. 100% was defined for APC generation by TM and all others were compared with TM. Data points represent the mean ± SD, for three independent measurements.
2.3 Conclusion

In conclusion, a one-pot/site-specific strategy for double modification of recombinant thrombomodulin via copper-free and sortase-mediated ligation (SML) without upsetting its anticoagulatory activity has been developed. Both reactions have advantages of mild reaction conditions and occurring in a single step without prior chemical modification of the target protein. We found that CFCC is more efficient for modification of TM with both large and small substrates. However, SML requires high concentration of protein-LPXTG and diglycine containing substrate to reach a satisfying reaction yield. Particularly, our results suggest that SML is more suited for protein modification with small molecules, such as fluorescent dye and biotin. In the case of some large substrates or the substrates with poor solubility, SML generates excess intermediate byproduct and gave low yield of product. In this study, we took the advantages of each ligation reaction to doubly modify the C-terminal of TM456 via one-pot reaction. We anticipate that the double-modified antithrombotic rTM456 with PEGylated and fluorescence-labeled will show an enhanced pharmacokinetic property with easy fluorescent probing feature in both in vitro and in vivo assay. We believe that this strategy is viable for modification of a variety of proteins with different molecules for elevated activity and stability, novel functions, easy bioassay capacity, and other specific properties of interest as well.

2.4 Materials and methods

2.4.1 Materials

All solvents and reagents were purchased from commercial sources and were used as received unless otherwise noted. pET DsbA Fusion System 39b, competent cells, and
kanamycin sulfate were purchased from EMD Chemicals (Philadelphia, PA). The mouse monoclonal antibody specific to human TM was purchased from COVANCE Corp. (Richmond, CA). 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 were obtained from Aniara. Anti-PEG monoclonal Ab E11 was purchased from Academia Sinica. DIBO-Alexa Fluor 647 was purchased from Invitrogen. DBCO-mPEG5000 was purchased from Click-Chemistry Tools. Synthesis of GlyGly-Biotin, GlyGly-Dansyl, and the expression of S. aureus sortase A were described in our previous report.13

2.4.2 Construction of plasmid pET39b-TM456AL

The gene encoding the EGF domain 4-6 of human thrombomodulin (corresponding to residues 345–473), with a C-terminal LPETG motif and an N-terminal human Factor Xa cleavage site (IEGRS), 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 before.20 The gene fragment was then inserted into the Sca I-Nco I sites of the pET39b vector containing an N-terminal DsbA enzyme fusion to a leader sequence containing His-tag to achieve the expression of plasmid pET39b-TM456AL.
The expression plasmid pET39b-TM\textsubscript{456}AL was transformed into \textit{E. coli} B834 (DE3) cells and expressed at 37 °C in M9AA medium (1 L) supplemented with 1 mM MgSO\textsubscript{4}, 0.1 mM CaCl\textsubscript{2}, 0.4 % (wt/v) glucose, 1 mg/L thiamine chloride, 35 mg/L kanamycin, and all proteinogenic amino acids. Upon reaching an OD\textsubscript{600} value between 0.8 and 1.0, methionine was removed from the media by centrifugation for 10 min at 4000 x g at 4 °C, and three times washes with 200 mL of M9 AA medium. The pellet was then resuspended in 1 L of the M9AA medium without methionine and incubated at 37 °C for 1 h to starve the cells followed by addition of L-azidohomoalanine to a final concentration of 40 mg/L. Protein expression was induced by 1 mM IPTG at 30 °C for 6 hours. The cell culture was then centrifuged at 6000 g for 10 min and the pellet was collected and kept in -20 °C until use.

### Figure 2.9
Amino acid sequence of DsbA-TM\textsubscript{456}AL fusion protein (359aa).
2.4.4 Purification of TM₄₅₆AL

The cell pellet 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 a 5 mL HiTrap Chelating column (GE Healthcare) charged with Ni²⁺ ions and eluted with 20 mM Tris, 0.5 M NaCl and 250 mM imidazole, pH 8.0. Pooled fractions containing recombinant TM were collected and then dialyzed against 20 mM Tris and 150 mM NaCl, pH 8.0, to afford DsbA/LPETG-tagged TM₄₅₆ fusion protein (DsbA-TM₄₅₆AL). The DsbA tag of fusion protein was removed by treatment with thrombin, and the released TM₄₅₆AL was then purified by using HiTrap Q chromatography.

2.4.5 Optimization of one-pot reaction conditions

The one-pot ligation reaction was optimized by using TM₄₅₆AL, SrtA, GlyGly-Bodipy and DIBO-Alexa Fluor 647 in different pH, temperatures, and concentrations in a 20 µL reaction system by fixing the concentrations of TM₄₅₆AL and Ca²⁺ at 10 µM and 5 mM, respectively. After 2 hours incubation, the reaction mixtures were loaded onto SDS-PAGE, and the fluorescent images of Bodipy (Ex/Em=488/520 nm) and Alexa Fluor 647 (Ex/Em=633/670 nm) were obtained by a Typhoon 9410 scanner. The fluorescence intensities were analyzed by Image J software, and the highest signal of each dye was defined as 100% and compared to the other results.

2.4.5.1 Optimizing reactant concentrations

SDS-PAGE conditions: various concentrations (10-2000 µM) of reactants were mixed with TM₄₅₆AL (10 µM), SrtA (5 µM) in 20 mM Tris-HCl containing 150 mM
NaCl and 5 mM Ca$^{2+}$, pH 8.0 (Buffer A). The total reaction volume was 20 µl and the reaction time was 2 h.

2.4.5.2 Optimizing reaction pH

SDS-PAGE conditions: GlyGly-Bodipy (400 µM) and DIBO-Alexa Fluor 647 (50 µM) were mixed with TM$_{456}$AL (10 µM), SrtA (5µM) in the buffers of various pH (50 mM Citrate buffer pH 3.0 to 6.0, 50 mM Hepes buffer pH 7.0, 20 mM Tris pH 8.0 and 50 mM sodium bicarbonate pH 9.0) in the presence of Ca$^{2+}$. The total reaction volume was 20 µL and the reaction time was 2 h.

2.4.5.3 Optimizing SrtA ratios

SDS-PAGE conditions: GlyGly-Bodipy (400 µM) and DIBO-Alexa Fluor 647 (50 µM) were mixed with TM$_{456}$AL (10 µM) and various concentrations (100 nM-10 µM) of SrtA in Buffer A. The total reaction volume was 20 µL and the reactions took place at 37 °C for 2 h.

2.4.5.4 Optimizing reaction temperatures

GlyGly-Bodipy (400 µM) and DIBO-Alexa Fluor 647 (50 µM) were mixed with TM$_{456}$AL (10 µM) and SrtA (5 µM) in Buffer A. The total reaction volume was 20 µL and the reactions took place at 4 °C, 20 °C and 37 °C for 2 h.
2.4.6 Preparation of double-modified TM\textsubscript{456}AL via one-pot reactions

A reaction mixture consisting of 10 μM TM\textsubscript{456}AL, 5 μM SrtA, 400 μM diglycine nucleophiles (GlyGly-Bodipy, GlyGly-Biotin or GlyGly-Dansyl) and 50 μM DBCO-PEG\textsubscript{5000}-OMe in Buffer A was incubated at 37 °C for 3 h. The CFCC and SML were quenched by addition of azidohomoalanine (20 mM stock solution) and EDTA (100 mM stock solution) to a final concentration of 1 mM and 10 mM, respectively. The successful ligations were then confirmed by SDS-PAGE followed by CBB staining, and fluorescent images were obtained by a Typhoon 9410 fluorescence scanner and UV light. For detection of Biotin-modified TM\textsubscript{456}AL, the SDS-PAGE gel was incubated with Streptavidin-Fluoresceine (100 μg/mL), and after 3 times washing with DDW, the fluorescent image was scanned (Ex=488, Em=526).

2.4.7 FITC labeling of sortase A

Briefly, 20 μL of freshly prepared 1 M sodium bicarbonate solution was added into 180 μL of sortase A (srtA), followed by 20 μL of FITC solution (10mg/mL in DMSO). The reaction mixture was gently vortexed at room temperature in the dark for 2 h. After the coupling reaction, the unreacted FITC was removed by HiTrap Desalting column (GE healthcare) to afford SrtA-Fluoresceine conjugate.

2.4.8 Protein C activation activity assay of TM\textsubscript{456}AL conjugates

The cofactor activity of TM\textsubscript{456}AL conjugates assessed by protein C activation assay as previously described with some modification. TM\textsubscript{456}AL conjugates from S7 were added into assay buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl\textsubscript{2}, pH 8.0)
containing 300 nM of Human protein C (PC) to a final concentration of 20 nM, and the reaction volumes were adjusted to 100 μL. 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 with shaking, the PC activation was terminated by addition of 30 μL human antithrombin III (1 mg/mL) and 2 μL heparin (10 U/mL) for 5 min at 37°C. The enzymatic activity of activated PC was measured by adding of chromogenic substrate H-D-Phe-Pip-Arg-pNa, 2HCl (0.5 mM). The increases in UV absorbance at 405 nm were measured after 20 min incubation from three independent experiments as shown in table II.

2.5 References


CHAPTER III

SITE-SELECTIVE INCORPORATION OF AZIDE FUNCTIONALITY INTO RECOMBINANT TM456 (TM3) FOR ITS BIO-ORTHOGONAL MODIFICATION

3.1 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. In particular, azide-based ligation reactions have been extensively explored for highly selective and biocompatible modification of proteins for biomedical applications. 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 a key step and versatile approach for site-selective protein modification research and applications.

Thrombomodulin (TM) is a membrane glycoprotein mainly expressed by vascular endothelial cells as a major anticoagulation component. 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. In the past decades, various domains of TM have been cloned and expressed for TM structural and functional study. 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. 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 and oriented immobilization through click chemistry. 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.\textsuperscript{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.\textsuperscript{16} Briefly, a TM of EGF-like 4-6 domains with an azidohomoalanine near C-terminus (rTM_{456}-N_{3}) was expressed via replacing methionine with azidohomoalanine from methionine auxotroph \textit{E. coli} cell. Then, site-selective conjugation of the rTM_{456}-N_{3} with liposome via Staudinger ligation and copper-free click chemistry (CFCC) afforded recombinant TM_{456}-liposome conjugates, which all 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 TM_{456} described above. In this study, we systemically investigated recombinant, enzymatic and chemical strategies to introduce azide functionality into TM_{456} site-selectively at either N-terminus or C-terminus (Figure 3.1). First, we investigated recombinant expression of recombinant TM_{456} with azide functionality near C-terminus by replacing methionine with azidohomoalanine from methionine auxotroph \textit{E. coli} cell. Second, we expressed TM_{456} 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 TM$_{456}$ via amidation chemistry. Finally, multiple azide functional groups were introduced into TM$_{456}$ via a tyrosine-selective three-component Mannich reaction. The azido-TM$_{456}$ derivatives obtained in all four methods were confirmed for site-selective modification and immobilization of recombinant TM through CFCC successfully.

**Figure 3.1** Multiple strategies for incorporating azide functionality into recombinant TM$_{456}$ for its bio-orthogonal modification applications.
3.2 Results

3.2.1 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. 17 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 III). 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 leaking. The designed gene fragment was inserted into pET28b (Novagen) plasmid and transformed
into *E. coli* B834 cell for expression (Figure 3.1). 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 III). 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 III. Preparation yield of pure (azido-)TM456 per 1 L of cell culture expression

<table>
<thead>
<tr>
<th></th>
<th>TM456 by Initial expression</th>
<th>Azido-TM456 by post-expression modification</th>
<th>Final TM456 amount per liter expression (mg/L)</th>
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</tr>
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<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>8.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Final pure azido-TM3-S amount was calculated based on the yield of SML.
Figure 3.2 Expression of TM3 and azdio-TM3 (A and B), and preparation of azido-TM3-S by SML (C and D). (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.

3.2.2 Incorporation of azide into TM3 via enzymatic method

First, we investigated an enzymatic method, sortase-mediated ligation (SML) to introduce azide group to TM<sub>456</sub> (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 an N-terminal poly-(glycine) molecule. 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-GGG 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. In this study, we use SML to introduce the azide group by using 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.

Figure 3.3 Preparation of azido-TM3-S by SML (A and B). (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.
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 2C, 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 3.3B, Lane 2), and both the unreacted TM3 and SrtA were in the eluate fraction (Figure 3.3B, 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 more 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 III). 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 3.4A, 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.

### 3.2.3 Incorporation of azide into TM3 via chemical methods

Chemical modification of native protein has been the main choice in the past.\textsuperscript{23} Most modification approaches aim at a special amino acid such as Lys and Cys, or N-terminal amine.\textsuperscript{24} 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 3.4A).

Recently, tyrosine becomes an attractive target for protein modification because of its relatively infrequent occurrence and ability to be modified without altering protein charge.⁴⁵, ⁴⁶ 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.⁴⁵ 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.⁴⁶ 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 3.4B). These results indicated that tyrosine-targeted methods can be used to incorporate azido functionality into the TM3 site-selectively.
Figure 3.4 SDS-PAGE and fluorescent image of azide modified TM3s and after reacted with DBCO-Cy5 (A and B. (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.

3.2.4 Protein C activation activity of azido-TM_{456}.

After successfully prepared four kinds of azido-TM_{456}s via multiple approaches above, we investigated the effect of the azide group on the activity of TM_{456}. 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-TM_{456}s 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 TM_{456} showed high APC generation
activities (Figure 3.5). Compared with unmodified TM3, azide modified TM$_{456}$ by site-selective methods (azido-TM$_2$, azido-TM$_3$-S and azido-TM$_3$-N) gave comparable activities, indicating that either terminus or near terminus modification of TM$_{456}$ by a single azide molecule does not influence the TM$_{456}$ activity. However, azido-TM$_3$-T showed a slightly decreased activity, which may be due to the multi-site modifications on tyrosine residues of TM$_{456}$ that affect either thrombin or PC interaction related with protein C activation.

![Protein C activation activities of free TM$_{456}$s](image)

**Figure 3.5** Protein C activation activities of TM3 and azido-TMs. Data points represent the mean ± SD, for three independent measurements. Control group contained no TM$_{456}$ at all.
3.2.5 Immobilization of azido-TM\textsubscript{456} on DBCO-coated glass

TM is 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\textsuperscript{27-32}. Compared with full TM, TM\textsubscript{456} 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-TM\textsubscript{456}s obtained above for immobilization on 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 3.6C) compared with FITC-TM2 (Figure 3.6B) that did not contain the azide group to react with DBCO. 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 3.6E, F, G), and no significant differences were observed among them. Further, protein C activation activities of these immobilized azido-TM\textsubscript{456}s were measured and compared with the unmodified TM\textsubscript{456}s (Figure 3.7).
Figure 3.6 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).
Figure 3.7 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.

3.3 Discussion

Azide is the key molecule in this 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 TM$_{456}$ 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-TM\textsubscript{456} is quite lower than native one (Table III). Therefore, in order to increase the total amount of azido-TM\textsubscript{456} production, we tried to use post-expression modification to introduce azide group to TM\textsubscript{456} 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\textsuperscript{17,33}. 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 \textit{et al.} utilized SML to generate protein thioester by peptide1-LPETG and G\textsubscript{n}-thioester, followed by native chemical ligation (NCL) to link to the peptide with N-terminal Cys\textsuperscript{34}. 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 PEG\textsubscript{3}-azide group to TM\textsubscript{456}, allowing the further protein modification by large, poor water soluble molecules through CFCC. Another feature is that fusion TM\textsubscript{456} and SrtA with a His-tag at the C-terminus facilitate 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 3.3). 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 III), which will facilitate site-selective modification of TM3 in large amount for practical applications.

3.4 Conclusion

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 variety of methods for introducing azide functionality into recombinant protein for its site-selective modification application, all of which can be applied to proteins of different interests.
3.5 Materials and methods

3.5.1 Materials

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 Aniara. 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).

3.5.2 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 before.35 The FLAG-TM3 gene was then transferred into pET-28b vector containing a C-terminal Histag between Ncol 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 OD$_{600}$ of 0.8 was reached, and then IPTG was added to a final concentration of 1 mM to induce the overexpression of TM$_{456}$ 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.

### 3.5.3 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 MgSO$_4$, 0.1 mM CaCl$_2$, 0.4 % (wt/v) glucose, 1mg/L thiamine chloride, 35 mg/L kanamycin and proteinogenic amino acids to the point that OD$_{600}$ 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.
3.5.4 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 Ni$^{2+}$ ions and eluted with buffer (20 mM Tris, 0.5 M NaCl and 250 mM imidazole, pH 8.0). Pooled fractions containing recombinant TM$_{456}$ were collected and then dialyzed against 20 mM Tris and 150 mM NaCl, pH8.0 to afford TM$_{456}$ or azido-TM$_{456}$ 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.

3.5.5 Incorporation of azide to TM3 via sortase-mediated ligation

TM3 (10 μM) was mixed with SrtA (5 μM) and diGly-PEG$_3$-azide (100 μM) in reaction buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl$_2$, 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-PEG$_3$-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.

3.5.6 Incorporation of azide to TM3 via amidation chemistry

NHS-PEG$_4$-azide was added to TM3 (10 μM) 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.

3.5.7 Incorporation of azide to TM3 via three-component Mannich reaction

200 μL of 4-Azidoanaline solution (5 μmol in 0.1 M phosphate buffer, pH 6.5,), 200 μL of formaldehyde solution (5 μmol in 0.1 M phosphate buffer, pH 6.5), and 200 μL 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-azidoanaline and formaldehyde were then removed by HiTrap Desalting column to obtain azido-TM3-T.

3.5.8 Fluorescent labeling of azido-TM₄56

To a solution of azido-TM₄56 (10 μM), DBCO-Cy5 was added to a final concentration of 20 μM. 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).

3.5.9 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.³⁶ 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 μL. 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 μL human antithrombin III (1 mg/mL) and 2 μL 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.

3.5.10 Immobilization of azido-TM456 on DBCO-coated glass

DBCO-coated glass was prepared by coating the aminosilanate glass slide with DBCO-PEG₄-NHS ester (S2). For immobilization of azido-TM2 via CFCC, FITC-labeled azido-TM2 (10 μM) 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. 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-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 μM) 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).

3.5.11 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. 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 μL of human antithrombin III (1 mg/mL) and 2 μL of heparin (10 U/mL) and incubated for 10 min at 37°C. 200 μL 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.
3.6 References


CHAPTER IV

SYNTHESIS AND CHARACTERIZATION OF LIPOSOMAL RECOMBINANT
THROMBOMODULIN CONJUGATE

4.1 Introduction

Liposome is a popular drug carrier for small drugs, proteins and genes with many features.\(^1,^2\) It has the function to enhance blood retention and provides protection for the encapsulated drugs due to the membrane structure of the vesicles which actually blocks the interaction between the drugs inside and outside environment. This kind of protection is no doubt can further enhance the biodistribution of drugs. Many liposome-type drugs including AmBisome (Astellas Pharma US, Inc., Deerfield, IL) and Doxil (Ortho Biotech, Horsham, PA), are successfully used in therapeutic application currently. Moreover, PEG can be covalently incorporated into liposome to improve the quality and efficiency of liposomes due to the lower immunogenicity and excellent biocompatibility of long chain PEG molecule.\(^3\) One significant advantage of liposome with PEG is the prolongation of
half-life of drug \textit{in vivo}, for instance, the half-life of Doxil is 100 times than free doxorubicin.\textsuperscript{4} Due to all these attributes, liposomal formulation is one of the most interesting area for drug delivery.

Besides the advantage of increasing half-life, the liposomal formulation of TM\textsubscript{456} could mimic the membrane protein nature of full TM, and therefore may facilitate to achieve better activity. Several attempts were made to incorporate TM into liposome. For example, full rabbit TM was directly constituted into liposome to enhance early syngeneic islet engraftment by reducing the instant blood-mediated inflammatory reaction (IBMIR),\textsuperscript{5} and improves early outcomes after intraportal islet transplantation.\textsuperscript{6} More recently, Zhang et al. in our lab reported a liposomal TM conjugate by using post-functionalization approach of liposome preparation, and this TM conjugate shows promising \textit{in vitro} antithrombotic activity.\textsuperscript{7}

In this study, liposome-TM\textsubscript{456} consisting of DSPE-PEG\textsubscript{2000}-TM\textsubscript{456} was designed and generated \textit{via} bio-orthogonal conjugation, copper-free click chemistry, and its \textit{in vitro} and \textit{in vivo} characterization were conducted as well. In order to prepare the long-circulation liposome, PEG\textsubscript{2000} was utilized, and the size of the liposome was monitored to ensure that it matched the size range of long-circulation liposome. The \textit{in vitro} protein C activity of liposome-TM\textsubscript{456} to confirm whether this new formulation affect TM\textsubscript{456} anticoagulant activity was investigated. Further, \textit{in vitro} plasma stability and \textit{in vivo} antithrombotic effect of the liposome-TM\textsubscript{456} were investigated with thrombin-induced thromboembolism mouse model. Finally, the prolongation of thrombin clotting time was
measure in a time-dependent manner *in vivo* and confirmed that the liposomal formulation of TM$_{456}$ did enhance its circulation time.

**Figure 4.1** Strategy for liposome-TM$_{456}$ synthesis and characterization. CFCC: copper-free click chemistry. DPPC: dipalmitoyl phosphatidyl choline. Chl: cholesterol. PC: protein C.

### 4.2 Results and discussion

#### 4.2.1 Synthesis of liposome-TM$_{456}$ conjugate *via* copper-free click chemistry

In general, three methods have been investigated to prepare liposome-protein conjugate (proteoliposome). One of them is the “post-insertion” method, which is normally used to prepare targeting liposome. It allows the protein only stay on the surface
of the liposome, and therefore saves the inner space of liposome.\textsuperscript{8-10} However, it requires a relatively high temperature, which sometimes reduces protein’s activity. Therefore, it can only be applied to those robust proteins. Another method is post-functionalization which attaches the proteins to the liposome surface by preparing the liposome first with anchor lipid followed by the conjugation of protein and anchor lipid. However, problems always arise from the cross-linking reactions and resulting aggregation of liposomes.\textsuperscript{11} Moreover, the liposome size sometimes alters after the conjugation and will affect the clearance behavior \textit{in vivo}.\textsuperscript{12} In order to maximally maintain the activity of TM\textsubscript{456} and the optimal size for long circulation time \textit{in vivo}, we selected the third method, direct liposome formation method, in which the protein-lipid is synthesized first then mixed with and other lipids directly to form liposome-TM\textsubscript{456}.

In this study, DSPE-PEG\textsubscript{2000}-TM\textsubscript{456} was prepared first from DSPE-PEG\textsubscript{2000}-DBCO and azido-TM\textsubscript{456} by copper-free click chemistry (CFCC). The PEG\textsubscript{2000} chain between the lipid and protein functions as “mushroom” conformation to form the stealth liposome. Stealth liposome provides enhanced circulation time in blood compared with the traditional liposome. On the other hand, the azido-TM\textsubscript{456} was prepared \textit{via} sortase-mediated ligation (SML) by expressing the LPETG motif containing TM\textsubscript{456}, with the catalysis of sortase A, the substrate GlyGly-PEG\textsubscript{3}-azide was conjugated to the C-terminal of TM\textsubscript{456} developed in Chapter III. Therefore, it is a site-specific modification of TM\textsubscript{456}, resulting in only one azide group on the C-terminal. Previous study revealed that SML offers the highest overall yield of azido-TM\textsubscript{456} comparing with other chemical modification methods or unnatural amino acid expression (incorporation of azide group to protein during expression). Further, SML provides a simple way to purify the reaction
mixture to obtain the pure azido-TM<sub>456</sub> from the unreacted TM<sub>456</sub>. Therefore, SML-mediated incorporation of azide facilities large amount of azido-TM<sub>456</sub> for the liposomal-TM<sub>456</sub> preparation, especially for the in vivo study.

After the reaction of azido-TM<sub>456</sub> and DSPE-PEG<sub>2000</sub>-DBCO, the new conjugate DSPE-PEG<sub>2000</sub>-TM<sub>456</sub> was confirmed by SDS-PAGE and western blot. A new band showed up around 35 kDa (Figure 4.2 Lane2) and the change of molecular weight was slightly higher than expected since the molecular weight of DSPE-PEG<sub>2000</sub>-DBCO is only around 3 kDa. This may be due to the hydrophilic nature change of TM<sub>456</sub> after the conjugation with DSPE-PEG<sub>2000</sub>. Further, the original band for pure azido-TM<sub>456</sub> disappeared completely, which indicated that all the azido-TM<sub>456</sub> reacted with DSPE-PEG<sub>2000</sub>-DBCO. To further confirm this band is the new conjugate lipid-TM<sub>456</sub>, western blot was performed by using anti-PEG antibody which recognizes PEG chain longer than 2000 units. It was clearly observed that the same new formed band in western blot (Figure 4.2 B), indicating that the new band is the DSPE-PEG<sub>2000</sub>-TM<sub>456</sub>.

![SDS-PAGE and western blot](image)

**Figure 4.2** SDS-PAGE and western blot of azido-TM<sub>456</sub> and DSPE-PEG<sub>2000</sub>-TM<sub>456</sub> conjugate. The western blot was performed by anti-PEG antibody which recognizes PEG chain in DSPE-PEG<sub>2000</sub>-TM<sub>456</sub> conjugate.
The liposome-TM$_{456}$ was then prepared with the DSPE-PEG$_{2000}$-TM$_{456}$, together with the other two lipids, DPPC and cholesterol. Briefly, the mixture of DPPC and cholesterol was dissolved in chloroform and dried to form the thin lipids layer. After re-suspended in PBS buffer and freeze-thaw processes, the DSPE-PEG$_{2000}$-TM$_{456}$ was added to the PBS solution containing DPPC and cholesterol, and incubated for 1 hour at room temperature with shaking. Then, the mixture was extruded by 400 nm and 100 nm polycarbonate filters, respectively. The plain liposome was prepared by the same process except by using DSPE-PEG$_{2000}$-DBCO instead of DSPE-PEG$_{2000}$-TM$_{456}$. The liposome conjugates were investigated by dynamic light scattering (DLS), since the size is a determining parameter for the half-life of liposome in vivo, and an optimal size for long circulation is around 100 nm. As shown in Table IV, the sizes of the liposomes with or without TM$_{456}$ are almost the same, which are 130 nm. It is in concordance with the extrusion by 100 nm filter and within the size range for long circulation in vivo.

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<th>Table IV. Liposome size measurement by DLS.</th>
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<td>Diameter (nm) a</td>
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<td>a. Mean ± standard deviation; n=3</td>
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4.2.2 Protein C activation activity of azido-TM$_{456}$ and TM$_{456}$ conjugates

In order to know whether the liposome modification to TM$_{456}$ affects TM$_{456}$’s anticoagulant activity, protein C activation activities of azido-TM$_{456}$ and TM$_{456}$-liposome conjugates were measured. The absorptions of activated protein C (APC) cleaved chromogenic substrates were measured at 405 nm and then compared. The rate of activating PC by thrombin is quite slow in the absence of TM$_{456}$ (Figure 4.3, column 1), while in the presence of azido-TM$_{456}$ and TM$_{456}$-liposome conjugates, the generation of APC was significantly enhanced (Figure 4.3, column 2-5). As shown in Figure 4.3 after the conjugation of DSPE-PEG$_{2000}$-DBCO and azido-TM$_{456}$, there was no significant change in PC activation activity, indicating that the lipidation didn’t hamper the TM$_{456}$ activity. As mentioned in the chapter III, the azido-TM$_{456}$ retained the same activity as TM$_{456}$ by using SML. These two results demonstrated that the site specific lipidation of TM$_{456}$ didn’t affect its anticoagulant activity, even using a two-step reaction involving SML and CFCC.

However, the activity of liposome-TM$_{456}$ showed around 30% lower than both azido-TM$_{456}$ and DSPE-PEG$_{2000}$-TM$_{456}$. This may be caused by the liposomal formulation of DSPE-PEG$_{2000}$-TM$_{456}$ entrapping a part of TM$_{456}$ facing inside the liposome. Therefore, this part of TM$_{456}$ was blocked by the bilayer of liposome to interact with thrombin and protein C in the solution. To test this speculation, the detergent, triton X-100 was used to break the liposome, therefore all the DSPE-PEG$_{2000}$-TM$_{456}$ had the access to the reagents in protein C activation assay. As expected, in the presence of detergent, the protein C activation activity of liposome-TM$_{456}$ increased to the same level as azido-TM$_{456}$. This suggested that the liposomal formulation didn’t affect the activity of TM$_{456}$, and the
difference of activities between the assay with and without triton X-100 should correspond to the TM$_{456}$ inside the liposome.

Figure 4.3 Protein C activation activity of azido-TM$_{456}$ and TM$_{456}$ conjugates. Triton X-100 was used to break the liposome and release all the DSPE-PEG$_{2000}$-TM$_{456}$. Data points represent the mean ± SD, n=3. Control group contained no TM$_{456}$ at all.

4.2.3 Plasma stability of azido-TM$_{456}$ and TM$_{456}$ conjugates

Plasma stability has significant implication in drug development. Drugs with poor plasma stability often show short half-lives, and further impact the distribution and efficacy of the drug. Blood contains an extensive number of enzymes, therefore, most plasma instability is due to the hydrolysis by these enzymes, especially proteases for
protein and peptide drugs.\textsuperscript{14-16} Due to this reason, the \textit{in vitro} plasma stability of azido-TM\textsubscript{456} and TM\textsubscript{456} conjugates were investigated. Briefly, azido-TM\textsubscript{456} and TM\textsubscript{456} conjugates were incubated with mouse plasma at 37 °C for different time periods, and the changes of protein concentration were measured by sandwich ELISA with anti-TM antibody and anti-FLAG antibody as capture antibody and detection antibody respectively. As shown in Figure 4.4, azido-TM\textsubscript{456} degraded to less than 40% after 3 hour, whereas DSPE-PEG\textsubscript{2000}-TM\textsubscript{456} and liposome-TM\textsubscript{456} presented much slower degradation. Even after 24 hours, there was still more than 50% of the DSPE-PEG\textsubscript{2000}-TM\textsubscript{456} remaining in the plasma. This should be due to the protection effect of the PEG\textsubscript{2000} molecule in between the lipid and protein. Long PEG chain is a well-known molecule to improve drug’s pharmacokinetic properties, mainly owing to a reduction of clearance rate by the kidney and proteolysis.\textsuperscript{17,18} As for this \textit{in vitro} assay, the prominent affect of the PEG\textsubscript{2000} chain as well as the lipid should be the steric hindrance\textsuperscript{19} which block the hydrolysis of TM\textsubscript{456} by those proteases in the plasma. Specifically, because of the exopeptidases existing in plasma, modification of N- or C- terminal of protein provides an efficient way to increase enzymatic stability.\textsuperscript{20} It was also observed that liposome-TM\textsubscript{456} had a longer \textit{in vitro} clearance time in plasma than DSPE-PEG\textsubscript{2000}-TM\textsubscript{456}. Besides the reason of PEG chain protection mentioned above, this enhancement of \textit{in vitro} stability in plasma may be because of the partial TM\textsubscript{456} facing inside the liposome, which cannot be accessed by the proteases, and leading to better stability than DSPE-PEG\textsubscript{2000}-TM\textsubscript{456}.
Figure 4.4 In vitro stability of azido-TM$_{456}$ and TM$_{456}$ conjugates in mouse plasma. Azido-TM$_{456}$ and TM$_{456}$ conjugates were incubated with mouse plasma at 37 °C for 0, 1, 3, 6, 12, 24 and 48 hours, and the concentration of azido-TM$_{456}$ and TM$_{456}$ conjugates were measured by sandwich ELISA by using anti-TM antibody as capture antibody and anti-FLAG antibody as detection antibody. Data represent mean ± standard deviation; n=3.

4.2.4 Antithrombotic effect of azido-TM$_{456}$ and TM$_{456}$ conjugates in thrombin-induced thromboembolism mouse model

The in vivo antithrombotic effect of liposome-TM$_{456}$ conjugate was tested in a thromboembolism mouse model. The thrombin-induced thromboembolism mouse model is a well-established assay to determine the antithrombotic effects of antithrombotics.$^{21,22}$ After injected of human thrombin, the mouse dies within 15 minutes due to acute fibrin
deposition within pulmonary microvascular system.\textsuperscript{21,22} Therefore, different doses of human thrombin were injected into C57Bl/6 mice, and the mortality was measured within one hour. It turned out that 75 U/mouse of human thrombin led to mortality larger than 80\%, and this dose was used to establish the thromboembolism mouse model.

Azido-TM\textsubscript{456} and liposome-TM\textsubscript{456} were injected into the mouse tail vein three minutes prior the injection of human thrombin. The control group injected PBS buffer only before human thrombin didn’t change the mortality, which is still 80\% (Figure 4.5). For azido-TM\textsubscript{456} three different doses (4.4 μg/mouse, 8.7 μg/mouse and 17.4 μg/mouse) were investigated. At a dose of 4.4 μg/mouse, there was almost no anti-thrombotic effect for the mice, since the mortality was still 80\%. While as the dose increased, the mortality decreased from 80\% to 20\% (Figure 4.5). In the group injected with 17.4 μg/mouse of azido-TM\textsubscript{456}, only one mouse was dead out of five. It was noticed that 17.4 μg/mouse corresponded to around 2-fold mol/L of the 75 U of thrombin injected. It has been reported that injection of recombinant human soluble thrombomodulin (TMD123) by 2.4-fold mol/L excess of thrombin saved all the mice from the acute thromboembolism.\textsuperscript{21} This suggested that synthesized azido-TM\textsubscript{456} can achieved a similar anti-thrombotic effect compared with TMD123.

Next, the anti-thrombotic effect of liposome-TM\textsubscript{456} were determined together with the plain liposome as a control. It turned out the plain liposome didn’t have any effect on this thromboembolism mouse model, still having 80\% mortality (Figure 4.5). By using the same dose of azido-TM\textsubscript{456} caused 20\% mortality, indicating that the liposome-TM\textsubscript{456} achieved the same anticoagulant effect as azido-TM\textsubscript{456} (Figure 4.5). Even though the \textit{in vitro} anticoagulant assay suggested that there was around 30\% reducing in activity of
unbroken liposome-TM_{456} than azido-TM_{456} due to the partial TM_{456} inside the liposome, the \textit{in vivo} anticoagulant effect of liposome-TM_{456} remained the same as azido-TM_{456}. This is probably because of the different \textit{in vivo} behaviors between free protein and liposome conjugated protein,\textsuperscript{23} especially the longer circulation time that may affect biodistribution. Moreover, it is demonstrated PEGlated lipids can be incorporated to the cell surface spontaneously,\textsuperscript{24-26} and thus the lipidated TM_{456} may be inserted to the cell membrane, especially endothelial cell by lipid fusion. This process can further enhance the anticoagulant activity of TM_{456}, since the lipid bilayer increases the binding affinity of TM/thrombin complex to protein C.\textsuperscript{7,27} Therefore, the total \textit{in vivo} anticoagulant effects of azido-TM_{456} and liposome-TM_{456} were different from the \textit{in vitro} PC activation activity, and detail mechanism will be investigated in the future.
Figure 4.5 Effect of azido-TM$_{456}$ and liposome-TM$_{456}$ on thrombin-induced thromboembolism in mice. Control group was injected with PBS buffer instead of azido-TM$_{456}$. Three different doses of azido-TM$_{456}$ were used, 4.4 µg/mouse, 8.7 µg/mouse, and 17.4 µg/mouse, whereas only the highest dose was applied for liposome-TM$_{456}$. The dose was calculated only based on protein component in the liposome-TM$_{456}$. Each group contained 5 mice, and the number of dead mice within 1 hour was counted.

4.2.5 Prolongation in thrombin clotting time of liposome-TM$_{456}$ conjugate

In order to determine liposome-TM$_{456}$ effect on thrombin clotting time (TCT) as an anticoagulant, both the *in vitro* and *in vivo* TCT was measured for pure azido-TM$_{456}$ and liposome-TM$_{456}$. Different concentrations of azido-TM$_{456}$ or liposome-TM$_{456}$ were mixed with plasma, and then human thrombin was added to trigger the clotting. For both azido-TM$_{456}$ and liposome-TM$_{456}$, increasing the concentration resulted in the increasing
of clotting time (Figure 4.6A), giving a direct relationship between the two. At concentrations of 0.001 mg/mL and 0.005 mg/mL, there was no difference in clotting time for azido-TM$_{456}$ and liposome-TM$_{456}$. But for higher concentrations, the difference between the two became more obvious. This may be because the concentrations below 0.005 mg/mL were too low to influence thrombin clotting time. For azido-TM$_{456}$, a concentration of 0.05 mg/mL doubled the clotting time, whereas twice of that concentration, 0.1 mg/mL of liposome-TM$_{456}$ reached the same antithrombotic effect (Figure 4.6A). The lower antithrombotic activity of liposome-TM$_{456}$ compared with azido-TM$_{456}$ was consistent with the APC activities, which was also due to the fact that a part of TM$_{456}$ was encapsulated in the liposome or facing the inside of the liposome, having no chance to react with thrombin and protein C in the plasma.

Next, the *in vivo* effect of azido-TM$_{456}$ and liposome-TM$_{456}$ on TCT in mouse at different time points was investigated. 3 mg/kg of azido-TM$_{456}$ or equivalent molar amount of liposome-TM$_{456}$ was injected to C57Bl/6 mouse through tail vein, and blood was collected at 0, 0.5, 1, 3, 6 hours. The blood contained no azido-TM$_{456}$ or liposome-TM$_{456}$ was also collected in order to measure the standard TCT as a control. TCT was then measured by mixing the human thrombin with the citrated mouse plasma. It turned out that the standard TCT was 24.8 ± 1.0 seconds. The prolongation of TCT was calculated by subtracting the standard clotting time from TCT in experiment groups. As shown in Figure 4.6B, the prolongation of TCT decreased from more than 40 seconds to less than 5 seconds in a time-dependent manner, which indicated the decrease of concentrations of azido-TM$_{456}$ or liposome-TM$_{456}$ in the plasma. For azido-TM$_{456}$, the prolongation of TCT decreased to less than 10 sec at 30 min, which is less than 15% of
the initial prolongation of TCT, suggesting that the concentration of azido-TM\textsubscript{456} dropped by a similar rate. This was the same as reported that E456 (epidermal growth factor-like domain 456) of human TM only has a half-life around 6-9 min in rats and monkeys.\textsuperscript{28}

While liposome-TM\textsubscript{456} demonstrated a very different blood profile, that showing a slower reducing in TCT prolongation compared with azido-TM\textsubscript{456}. At 30 min, the prolongation of TCT was still around 38 sec, which was almost five times longer than the one of azido-TM\textsubscript{456}. It indicated that there was more of TM\textsubscript{456} in liposomal formulation than free protein at 30 min, since there was a direct relationship between concentration and TCT. Till 6 hour, the prolongation of TCT dropped to around 8 sec which is the same level of azido-TM\textsubscript{456} at 30 min. It suggested that liposomal formulation enhanced the circulation time and elongated the time of anticoagulant effect of TM\textsubscript{456}.

TM\textsubscript{456} is a small protein with anticoagulant activity and only contains domain EGF456. It is different than human soluble TM which has anticoagulant activity as well as the coagulant activity via the thrombin activatable fibrinolysis inhibitor (TAFI) activation by domain EGF3-6. Therefore TM\textsubscript{456} has no coagulant activity and will be a potential candidate as pure anticoagulant. However, the main drawback of TM\textsubscript{456} is the short half-life, only 6-9 min in rats and monkeys,\textsuperscript{28} and this disadvantage limits the application of TM\textsubscript{456} as a protein drug. Half-life of only few minutes is not sufficient enough for drug to reach the target tissues or organs. This is commonly caused by the fast renal clearance related with the hydrophilic nature and the small size of the protein drug, or the enzymatic degradation by enzymes in blood.\textsuperscript{20} After incorporated the TM\textsubscript{456} into liposome, it showed an increase in half-life based on the longer effect of prolongation of TCT, still with the same \textit{in vitro} protein C activation activity. Several factors contributed
to this improvement in its half-life. First of all, the conjugation of long PEG chain and lipid at the C-terminal of TM$_{456}$ protected the degradation by enzyme in blood. Secondly, after the incorporation of DSPE-PEG$_{2000}$-TM$_{456}$ into liposome, a part of TM$_{456}$ was buried inside the liposome, which is also a kind of protection for this part of TM$_{456}$ by the lipid bilayer. Most importantly, the liposomal formulation altered the clearance pathway and resulted in a prolonged anticoagulant effect in mouse.
Figure 4.6 Prolongation of TCT. (A) Effect of azido-TM$_{456}$ and liposome-TM$_{456}$ on increasing thrombin clotting time (TCT) \textit{in vitro} at different concentrations. The concentration was calculated only on protein content. (B) Prolongation of TCT (clotting time increasing compared with pure mouse plasma) after injecting 3 mg/kg azido-TM$_{456}$ or equivalent molar amount of liposome-TM$_{456}$ to mice at different time points. Data points represent mean ± standard deviation, n=4, and two mice for each time point.
4.3 Conclusion

In summary, a lipidated TM$\text{_{456}},$ DSPE-PEG$\text{_{2000}}$-TM$\text{_{456}}$ was successfully synthesized by azido-TM$\text{_{456}}$ and DSPE-PEG$\text{_{2000}}$-DBCO via copper-free click chemistry. Then liposome-TM$\text{_{456}}$ was fabricated by using the DSPE-PEG$\text{_{2000}}$-TM$\text{_{456}}$ via direct liposome formation method, and the size of the obtained liposome was around 130 nm. This liposomal formulation of TM$\text{_{456}}$ retained the same protein C activation activity as the unmodified TM$\text{_{456}},$ and turned out to be much more stable in plasma than azido-TM$\text{_{456}}$ and even DSPE-PEG$\text{_{2000}}$-TM$\text{_{456}}$. Liposome-TM$\text{_{456}}$ also showed a comparable anticoagulant effect in thrombin-induced thromboembolism mouse model by decrease the mortality from 80% to 20%. Moreover, liposome-TM$\text{_{456}}$ enhanced the circulation time due to the fact that the prolongation of TCT decreased dramatically slowly than free azido-TM$\text{_{456}}$.

4.4 Materials and methods

4.4.1 Materials

C57BL/6 Mouse (6-8 weeks) were purchased from Charles River Laboratories International, Inc.. Mouse plasma and human plasma were purchased from Innovative Research (Novi, MI). Human protein C, human thrombin and human antithrombin III were obtained from Haematologic Technologies Inc.. Chromogenic thrombin substrate BIOPHEN-CS01 was obtained from Aniara. DPPC and DSPE-PEG$\text{_{2000}}$-DBCO were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Sigma-Aldrich (St.Louis, USA). Antibody coating buffer, Neptune block, Neptune sample diluent, ELISA wash buffer, TMB 1-component HRP microwell substrate (SUBT)
and stop solution for TMB substrates (STOPT) were purchased from ImmunoChemistry Technologies, LLC (Bloomington, MN). The mouse anti-TM monoclonal antibody PBS-01 was purchased from abcam (Cambridge, MA). THE™ DYKDDDDK Tag Antibody [Biotin], mAb, Mouse and Streptavidin-HRP were purchased from GenScript, USA, Inc (Piscataway, NJ). Anti-PEG monoclonal antibody E11 was purchased from Academia Sinica.

4.4.2 DSPE-PEG$_{2000}$-TM$_{456}$ preparation and western blot for identification

Azido-TM$_{456}$ and DSPE-PEG$_{2000}$-DBCO were mixed in reaction solution (20 mM Tris and 150 mM NaCl, pH 7.8) at final concentration of 10 µM and 200 µM respectively. The reaction solution was incubation at room temperature for overnight.

DSPE-PEG$_{2000}$-TM$_{456}$ was separated in a 15% SDS–polyacrylamide gel under reducing conditions and transferred onto a polyvinylidene difluoride membrane by Trans-Blot® Turbo™ Transfer system (Life science research, Hercules, Ca). After transfer, the membrane was blocked in 5% non-fat milk prepared in TBS/Tween 20 (0.05%) at room temperature for 1 h with gentle shaking. Primary anti-PEG monoclonal antibody E11 (1:1000) prepared in TBS/Tween 20 (0.05%), 2% non-fat milk (10 ml) was applied to the membrane and then incubated overnight at 4°C. After the incubation, the membrane was then washed with TBS/Tween 20 (0.05%) (4×15 minutes). Then, a secondary antibody (goat anti-mouse HRP 1:2000) prepared in TBS/Tween 20 (0.05%) was applied to the membrane for a further 1 h at room temperature incubation with shaking. Again, the membrane was washed with TBS/Tween 20 (0.05%) (4×15 minutes) and protein bands
were visualized using enhanced chemiluminescence (ECL) solution (GE Healthcare Bio-
Sciences, Pittsburgh, PA).

4.4.3 Liposome-TM₄₅₆ preparation

DSPC (15 mg, 20.43 μmol) and, cholesterol (4 mg, 10.2 μmol) were dissolved in 3.0 mL chloroform. The lipid mixture was dried onto the wall of a 100 mL round-bottom flask by removing the solvent gently using a rotate evaporator under reduced pressure followed by placing the vessel under vacuum overnight to form a thin lipid film on the flask wall. Then the lipid film was resuspended with 2 mL PBS to form a multilamellar vesicle suspension. Ten freeze-thaw cycles using liquid N₂ followed by immersion in a 65 ºC water bath were performed. 30 nmol of DSPE-PEG₂₀₀₀-TM₄₅₆ was added to the PBS solution containing DPPC and cholesterol, and incubated for 1 hour in room temperature with shaking. Then the mixture was extruded through polycarbonate membranes (Millipore size from 400 nm and 100 nm) to produce small unilamellar vesicles. The plain liposome was prepared in a similar method by replacing the DSPE-PEG₂₀₀₀-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO.

The size of plain liposome and liposome-TM₄₅₆ were determined by DLS (dynamic light scattering), using 90plus particle size analyzer (Brookhaven Ins. Co., USA). All measurements were performed at 25 ºC with a measuring angle of 90° to the incident beam. The values presented were the mean of three replicate samples together with the standard deviation.
4.4.4 Protein C activation activity assay

The cofactor activities of azido-TM$_{456}$ and TM$_{456}$ conjugates were assessed by protein C activation assay. Azido-TM$_{456}$ and TM$_{456}$ conjugates obtained from above were added into assay buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl$_2$, 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 μL. 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 μL human antithrombin III (1 mg/mL) and 2 μL 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.

4.4.5 Stability of azido-TM$_{456}$ and TM$_{456}$ conjugates in mouse plasma

Azido-TM$_{456}$ or TM$_{456}$ conjugates were mixed with mouse plasma to a final concentration of 50 μg/mL and incubated in 37°C. Samples were taken out at specified time points, and the concentrations of azido-TM$_{456}$ and TM$_{456}$ conjugates were measured by sandwich ELISA.

The capture antibody, Anti-thrombomodulin mAb (5 μg/mL) in Antibody coating buffer was allowed to bind overnight at 4 °C to high-binding EIA/RIA 8-well strips. Wells were then washed twice with ELISA wash buffer and blocked with Neptune block for 10 h at room temperature. TM$_{456}$ standards [0–40 ng/mL] were prepared in Neptune sample diluent. TM$_{456}$ derivatives samples were prepared identically to TM$_{456}$ standards.
TM₄₅₆ standards in plasma was prepared by spiking with mouse plasma at a concentration of 8 µg/ml, followed the dilution by Neptune sample diluent to reach the target concentration [0–40 ng/mL]. Azido-TM₄₅₆ and TM₄₅₆ conjugates samples in mouse plasma for stability study were prepared identically to TM₄₅₆ standards in plasma. Standards and samples were incubated overnight at 4 °C, washed 4 times for 5 min each. Then, the detection antibody, FLAG-Tag Antibody [Biotin] mAb was added to each well and incubated for 2 h at room temperature. After 4 times washes for 5 min each, Streptavidin-HRP was added to each well and incubated for 1 h at room temperature. After washes, TMB substrate was added for 30 min, and stop solution was added to terminate the reaction. OD at 450 nm was measured, and azido-TM₄₅₆ and TM₄₅₆ conjugates concentrations were then extrapolated from the linear equation based on the corresponding standard curve.

4.4.6 Antithrombotic effect of azido-TM₄₅₆ and TM₄₅₆ conjugates in thrombin-induced thromboembolism mouse model

Human thrombin was injected into the lateral tail vein of mice at 75 U/mouse to reach a mortality of larger than 80%. Three minutes before the injection of human thrombin, azido-TM₄₅₆ and TM₄₅₆ conjugates with indicated concentrations were injected to the lateral tail vein. Control mice received PBS buffer or plain liposome in PBS buffer. The number of dead mice within 1 h was counted, and the mice not killed by the thromboembolism were euthanized after 1 h.
4.4.7 Thrombin clotting time

C57BL/6 Mice were dosed with 3mg/kg azido-TM$_{456}$ and TM$_{456}$ conjugates through the tail vein. At each specified time point two mice from each treatment group were sacrificed, followed by immediately blood collection in cardiac puncture. The blood samples were then mixed with 3.2% buffered citrate sodium citrate medium (9 parts blood, 1 part anticoagulant). The collected blood was then centrifuge at 1500 g for 10 min to obtain the plasma. 100 μL of the plasma was mixed with 200 μL of 5 U/mL of human thrombin to initiate the clotting, and the TCT was measured by BBL fibrometer (Becton-Dickinson, Cockeysville, MD). Standard TCT was obtained by using the mouse plasma without azido-TM$_{456}$ or TM$_{456}$ derivatives.

For the concentration dependent TCT assay, 25 μL of azido-TM$_{456}$ or TM$_{456}$ conjugates with different concentrations were mixed with 75 μL human plasma to obtained final concentrations of 0.001 mg/mL, 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.15 mg/mL for protein content. Then the clotting was triggered by adding 200 μL of 5 U/mL of human thrombin.

4.4.8 Calculation of concentrations

Concentrations and doses were calculated based on protein content using the same molecular weight (16 kDa) for TM$_{456}$, DSPE-TM$_{456}$ and liposome-TM$_{456}$. Hence, at a given mg/mL concentration or mg/kg dose, equimolar amounts of the molecules were used.
4.5 References


CHAPTER V

SUMMARY

Endothelial thrombomodulin (TM) plays an important role in coagulation pathway as a cofactor of thrombin and it coverts thrombin’s procoagulant activities to anticoagulant activities. Besides, protein C is activated by thrombin and TM complex to generate activated protein C as an anticoagulant. A recombinant human soluble TM has been approved as drug for treating DIC, whereas this rhsTM has other biological functions including procoagulant activity via TAFI activation by domain EGF3-6 other than anticoagulant activity. Only domain EGF456 (TM_{456}) is a pure anticoagulant candidate, since the minimum domain for protein C binding and activating is EGF456. However, the major drawback of this protein is the short half-life, which is only 6-9 min.

Liposomes have been extensively studied as cell surface model as well as carrier for delivering small drugs, proteins and genes to the body. Particularly, incorporation of
protein drug with stealth liposome enhances the blood circulation time. Besides, the liposomal formulation of TM\textsubscript{456} could mimic the membrane protein nature of cell membrane TM, and thus achieve better activity. Therefore, the goal of this study is to conjugate TM\textsubscript{456} with PEG or liposome to produce novel and potential antithrombotic drug.

Firstly, a one-pot/site-specific strategy for double modification of recombinant TM via copper-free click chemistry and sortase-mediated ligation (SML) was developed. Both reactions have advantages of mild reaction conditions and occurring in a single step without prior chemical modification of the target protein. It turned out that CFCC is more efficient for modification of TM with both large and small substrates. However, SML requires high concentration of diglycine containing substrate to reach a satisfying reaction yield. Particularly, the results suggested that SML is more suited for protein modification with small molecules, such as fluorescent dye and biotin. For large substrates or the substrates with poor solubility, SML generates acyl enzyme intermediate as byproduct and thus reduced yield of product. Therefore, by taking the advantage of each ligation reaction, CFCC is the choice for PEGylation and SML for all the other fluorescent probes to prepare the double modification protein conjugates. Moreover, both sites of the modification are in C-terminal of TM\textsubscript{456}, and this kind of site-specific modification didn’t affect the bioactivity of TM\textsubscript{456}. These results indicated that this strategy is viable for modification of a variety of proteins with different molecules for elevated activity and stability, novel functions and other specific properties of interest as well.
Secondly, a multiple strategies for site-selectively introducing azide functionality into recombinant TM\textsubscript{456} were developed. TM\textsubscript{456} is a small protein containing 133 amino acid while has both thrombin and protein C binding domain. Therefore, in order to maximally retain its antithrombotic activity, the best choice is to modify the N- or C-terminal of TM\textsubscript{456}. By direct recombinant expression with unnatural amino acid, a near C-terminal azido-TM\textsubscript{456} was obtained; by SML, a C-terminal azido-TM\textsubscript{456} was obtained; by amidation chemistry, an N-terminal azido-TM\textsubscript{456} was obtained. All of these modification resulted in azido-TM\textsubscript{456} bearing a single azide molecule, and from the protein C activation assay, the activities of these TM\textsubscript{456} conjugates unchanged, indicating the N- or C- terminal modification is suitable for TM\textsubscript{456}. In the contrast, the multiple modifications by targeting tyrosine retained less than 80% of the activity. Overall, SML of recombinant protein affords the highest overall yield for incorporating azide functionality into recombinant TM\textsubscript{456} at the C-terminus since the key protein expression step uses all natural amino acids. This study provides variety of methods for introducing azide functionality into recombinant protein for its site-selective modification application, all of which can be applied to proteins of different interests.

Thirdly, liposome-TM\textsubscript{456} was successfully prepared via direct liposome formulation method by using lipidated TM\textsubscript{456}, DSPE-PEG\textsubscript{2000}-TM\textsubscript{456}, which is synthesized from the conjugation of azido-TM\textsubscript{456} (prepared by SML) and DSPE-PEG\textsubscript{2000}-DBCO. This liposome-TM\textsubscript{456} offered many new features compared with the free azido-TM\textsubscript{456} and retained the similar protein C activation activity. First of all, liposome-TM\textsubscript{456} turned out to be much more stable than azido-TM\textsubscript{456} and even DSPE-PEG\textsubscript{2000}-TM\textsubscript{456} in \textit{in vitro} plasma stability assay. Secondly, liposome-TM\textsubscript{456} showed a comparable
anticoagulant effect in thrombin-induced thromboembolism mouse model by reducing the mortality from 80% to 20%. Moreover, liposome-TM$_{456}$ showed enhanced circulation time. Overall, liposome-TM$_{456}$ provides a strategy for novel candidate for antithrombotic agent development.
CHAPTER VI
FUTURE DIRECTION

In this study, recombinant thrombomodulin (rTM)-based antithrombotic agents that mimic the native endothelial TM’s structure have been successfully developed. The \textit{in vitro} and \textit{in vivo} antithrombotic effect of liposome-TM$_{456}$ as well as the \textit{in vitro} stability was investigated, and it showed promising anticoagulant activity. Continually, there are still several prospects to be explored in order to study the detail mechanism of liposome-TM$_{456}$ \textit{in vivo}. Further investigations should be carried out to study its pharmacokinetics profile. The following researches are expected to be conducted in the future.

\textbf{6.1 Effect of liposome-TM$_{456}$ on bleeding time}

Many antithrombotic drugs including heparin showed very excellent anticoagulant activity, whereas most of them are faced with one severe disadvantage, the
risk of bleeding. This drawback leads to closely monitor for the patients taking these drugs, which is inconvenient to both patients and physicians and costly. Therefore, bleeding time determination is another important criterion to evaluate anticoagulant drug. To measure the bleeding time for liposome-TM$_{456}$ will give the information of how it affects the bleeding even though most reports mentioned that TM can efficiently reduce the formation of thrombosis without prolonging bleeding time. A simple way to determine bleeding time is mouse tail bleeding assay by measuring the blood volume collected from the amputated mouse tail.

6.2 Evaluation of pharmacokinetics and biodistribution of liposome-TM$_{456}$ in rats

Liposome can be used to protect the encapsulated drug from the degradation as well as increase the half-life. However, the changes brought by liposome are not always positive, and sometimes new sides effects may generated. Therefore, it is very important to monitor the pharmacokinetics (PK) and biodistribution of liposome-protein conjugates to understand and predict their efficacy and side effects. PK study includes determination of drug concentrations in all major tissues after drug administration for different periods of time. For liposome-TM$_{456}$, sandwich ELISA has the advantage to measure the conjugates concentration or the accumulation in different tissues.