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Bio-Inspired Liposomal Thrombomodulin Conjugate through Bio-Orthogonal Chemistry

Hailong Zhang¹, Jacob Weingart¹, Rui Jiang¹, Jianhao Peng², Qingyu Wu^{1,2}, and Xue-Long Sun^{1,2}

¹Department of Chemistry, Cleveland State University, Cleveland, OH 44115

²Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195

Abstract

We report the synthesis of bio-inspired liposomal thrombomodulin (TM) conjugates by chemoselective and site-specific liposomal conjugation of recombinant TM at C-terminus. TM is an endothelial cell membrane protein that acts as a major cofactor in the protein C anticoagulant pathway. To closely mimic membrane protein structural features of TM, we proposed membrane-mimetic re-expression of recombinant TM onto liposome. A recombinant TM containing the EGF-like 456 domains and an azidohomoalanine at C-terminus was expressed in *E. coli*. Conjugation of the recombinant TM onto liposome *via* Staudinger ligation and copper-free click chemistry were investigated as an optimal platform for exploring membrane protein TM's activity, respectively. The bio-inspired liposomal TM conjugates were confirmed with Western blotting and protein C activation activity. The recombinant TM-liposome conjugates showed a 2-fold higher k_{cat}/K_m value for protein C activation than that of the recombinant TM alone, which indicated that the lipid membrane has a beneficiary effect on the recombinant TM's activity. The reported liposomal protein conjugate approach provides a rationale design strategy for both studying membrane protein TM's functions and generating a membrane protein TM-based anticoagulant agent.

INTRODUCTION

Thrombomodulin (TM) is an endothelial anticoagulant protein that contributes to local hemostatic balance by modulating the activity of thrombin from a procoagulant to an anticoagulant protease.^{1–4} When bound to TM on the endothelial surface, thrombin is unable to cleave fibrinogen or activate platelets. Instead, it becomes a potent activator of protein C. Subsequently, the activated form of protein C (APC), an anticoagulant protease, selectively inactivates coagulation factors Va and VIIIa, providing an essential feedback mechanism in preventing excessive coagulation. Thrombotic disorders have been found to result from relative deficiency of TM.⁵ It also has been reported that reduction in TM activity, induced by a targeted point mutation or injection of anti-TM antibodies in mice, makes the animals more susceptible to thrombogenic challenges.^{6,7} In the past decades, various recombinant TM proteins consisting of extracellular portions have shown potential anticoagulant activity.^{8–13} One such example is recombinant human soluble TM α (rhsTM α or ART-123), which was recently approved in Japan to treat patients with disseminated intravascular coagulation (DIC).^{13,14}

Correspondence to: Xue-Long Sun.

Cleveland, OH 44115 Phone: (216) 687-3919 Fax: (216) 687-9298 x.sun55@csuohio.edu.

The structural domains of TM necessary for protein C activation have been resolved. The fifth and sixth EGF-like domains of TM represent the thrombin binding site, while the fourth EGF-like domain is responsible for protein C activation.^{2,3} On the other hand, TM's third EGF-like domain contributes to procoagulant activity of TM by accelerating the proteolytic activation of thrombin-activatable fibrinolysis inhibitor (TAFI) by thrombin.¹⁵ Activated TAFI (TAFIa) cleaves C-terminal lysine and arginine residues from partially degraded fibrin. The removal of these positively charged residues suppresses the ability of fibrin to catalyze plasminogen activation, thereby delaying clot lysis.¹⁶ Therefore, recombinant TM consisting of the entire extracellular portion has both procoagulant and anticoagulant activities and thus would be less effective as an anticoagulant agent. Consequently, recombinant TM containing the EGF-like domains 456 (rTM₄₅₆) has been considered to be a sole anticoagulant candidate.^{17–20} However, rTM₄₅₆ has a very short half-life (6–9 min) in animals, as compared to that of 5 h for rhtTM.²¹ Therefore, the pharmacokinetic properties of rTM₄₅₆, like many other potential protein therapeutics, limits its therapeutic applications.

Since TM is a membrane protein, it would be logical to develop a membrane mimetic conjugate of TM to mimic the native endothelial antithrombotic mechanism associated with cell membrane lipid components, thereby creating a more efficacious agent than current soluble TM without the membrane domain.²² Liposomes have been extensively studied as cell membrane models and as carriers for delivering vaccines, enzymes, drugs, and genes to sites of action.²³ In this study, we envisioned that liposomal rTM conjugates containing rTM₄₅₆ may provide a rational design strategy for facilitating studies of membrane TM's functions while generating an optimal platform for exploring membrane protein TM-based anticoagulant agent with acceptable pharmacodynamics and pharmacokinetics (Figure 1).

In the past, the production of protein-conjugates, to serve as effective biotherapeutics, was hindered by nonspecific conjugation reactions, resulting in a variety of isoforms, which potentially eliminate the protein's proper activity for the intended use. For example, PEGylation usually involves a reaction to available lysine amino groups of protein, some of which may be within or near a bioactive site. Thus, most protocols are nonspecific and result in a loss of protein activity.^{24,25} Recent advanced protein engineering permits the inclusion of unique attachment sites within recombinant protein for numerous applications such as protein functional studies and bioconjugation applications. For example, the introduction of chemically unique groups into proteins by means of non-natural amino acids allows for site-specific functionalizations.^{26,27} Three highly selective reactions: the Staudinger ligation,²⁷ Cu(I)-catalyzed azide-alkyne cycloaddition, known as click chemistry,²⁸ and strain-promoted azide-alkyne cycloaddition, known as copper-free click chemistry²⁹ have been developed so far for selective bioconjugation. Chaikof and co-workers reported an azido-containing rTM construct for the site-specific PEGylation *via* Staudinger ligation, which aimed to increase the serum half life time of rTM.²⁰ In addition, site-specific immobilization of the rTM derivative at the C-terminus through click chemistry with a suitably engineered alkyne PEG glass slide was also demonstrated.³⁰ The click chemistry has become of great use in modifying proteins and other macromolecules.³¹ However, a disadvantage of this reaction is the possible presence of residual copper, which can be potentially toxic, in the product intended for biological application.³² Recently, to avoid the use of the potentially toxic copper catalyst, the copper-free click chemistry has emerged as a popular bioorthogonal ligation strategy.³³ It has been used as a versatile chemistry for biomolecule modification,³⁴ cell surface,³⁵ and liposome³⁶ modification applications. Overall, the click chemistry was found to be most efficient for detecting azides in protein samples but was not compatible for making bioconjugates for therapeutic application due to the toxicity of the reagents. Both the Staudinger ligation and the copper-free click chemistry using optimized cyclooctynes are effective and compatible for bioconjugation.³⁷ In this study, we explored regio- and chemoselective liposomal modification of a recombinant TM₄₅₆ derivative at the

C-terminus through Staudinger ligation and copper-free click chemistry, respectively. The bio-inspired liposomal recombinant TM conjugates are expected to have enhanced pharmacodynamic and pharmacokinetic properties through membrane mimetic tactic (Figure 1).

MATERIALS AND METHODS

Materials and Reagents

All solvents and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Deionized water was used as a solvent in all experiments. Human thrombomodulin cDNA clone was from ATCC (Manassas, VA), pET Dsb Fusion System 39b, competent cells, and kanamycin sulfate were from EMD Chemicals (Philadelphia, PA). Phusion® High-Fidelity PCR Kit, BamHI-HF, KpnI-HF, T4 DNA ligase and alkaline phosphatase were from New England Biolabs (Ipswich, MA). All plasmid purification kits were from QIAGEN Inc. (Chatsworth, CA). *E. coli* strain B834 (DE3), plasmid pET-39b(+), S-Tag Rapid Assay kit, and Site-Specific Enterokinase Cleavage and Capture kits were from Novagen (Madison, WI). The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA). Synthetic oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Purified recombinant human PC and human thrombin were from Haematologic Technologies Inc. (Essex Junction, VT). Human anti-thrombin, recombinant human TM and chromogenic substrate Spectrozyme PCa were from American Diagnostica Inc. (Stamford, CT). L-azidohomoalanine was from AnaSpec Inc. (Fremont, CA). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-*sn*-glycero-3-phosphoethanol-amine-N-[amino(polyethylene glycol)2000] (NH₂-PEG₂₀₀₀-DSPE) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (mPEG₂₀₀₀-DSPE) were from Avanti Polar Lipids (Alabaster, AL). DBCO-PEG₄-NHS ester was from Click Chemistry Tools (Scottsdale, AZ). Glyco-Staining kit was from Geno Technology (St Louis, MO). Cholesterol, imidazole, sodium azide, *N,N*-dimethylformamide and other chemicals were purchased from Sigma-Aldrich (USA). Triphenylphosphine (TP) PEG₃₄₀₀-DSPE was synthesized as described previously.³⁸

Synthesis of DBCO-PEG₂₀₀₀-DSPE

To a dry 25 mL round bottom flask containing a stir bar, DSPE-PEG₂₀₀₀-NH₂ (94.4 mg, 0.034 mmol) was added and the flask was then equipped with a 3-way stopcock and placed under vacuum for 20 min. The sealed flask, equipped with a balloon, was backfilled with Ar(g) and then 3 mL of dry chloroform was added *via* syringe to dissolve the contents under stirring. Then, *via* syringe, triethylamine (0.05 mL, 0.36 mmol) was added. After 30 min, while stirring, DBCO-PEG₄-NHS ester (20 mg, 0.029 mmol) dissolved in 1 mL of dry chloroform was added *via* syringe dropwise slowly. The reaction mixture was stirred under Ar(g) at room temperature and was monitored *via* TLC (MeOH:CHCl₃ 1:10). After 48 h, the reaction mixture was concentrated under reduced pressure and the residue was purified *via* silica gel column chromatography using methanol/chloroform (1:10) as eluting solvent to afford pure DBCO-PEG₂₀₀₀-DSPE (77 mg, 79% yield). ¹H NMR (CDCl₃, 600 MHz) δ: 8.06 (s, 3H), 7.68 – 6.6 (d, 8H), 5.21 (s, 1H), 5.13 (d, 2H), 4.35 (d, 2H), 4.21 (t, 2H), 3.99 (m, 6H), 3.73 – 3.55 (m, 204H), 3.10 (t, 1H), 2.70 (t, 2H), 2.47 (t, 2H), 2.29 (t, 4H), 1.59 (t, 4H), 1.35 (t, 4H), 1.26 (t, 52H), 0.88 (t, 6H). ¹³C NMR (CDCl₃, 75 MHz) δ: 173.4, 173.0, 136.1, 132.1, 129.1, 128.6, 127.8, 125.6, 108.0, 93.2 (alkyne), 70.6 (PEG), 67.2, 55.5, 45.7, 39.1, 36.8, 34.1, 32.0, 29.71, 24.9, 22.7. FTIR (cm⁻¹): 3490, 2915, 2870, 2850, 2091, 1714, 1651, 1542, 1466, 1349, 1249, 1092, 948, 843, 720.

Plasmid constructs

Recombinant TM₄₅₆ was prepared by expressing plasmid TM₄₅₆-N₃ in *E. coli*. methionine auxotroph B834 (DE3). To encode thrombomodulin EGF 4-6 domains, forward primer: 5' CGC GGA TCC CGA CCC GTG CTT CAG A 3' and reverse primer: 5' GTT GCA AAA CAG CTG GCA CCT GTG 3' were used to create a mutant with C-terminal methionine based on human thrombomodulin cDNA, in which unnatural amino acid could be incorporated into recombinant proteins to provide an azide group for chemoselective modification. To remove extra methionine in other sites of the mutant and to improve resistance to oxidative inactivation and enzymatic activity, a point mutation was created to switch methionine to leucine at position 388 by using forward primer 5' CAC AGG TGC CAG CTG TTT TGC AAC 3' and reverse primer 5' CGC GGA TCC GAT TAC ATA CC C CCC AC 3'. PCR fragments containing the mutant sequences were ligated into pET 39 b(+) to make the expression plasmid TM₄₅₆GGM. The sequence of each mutant plasmid was confirmed by DNA sequencing.

Mutant TM₄₅₆ expression and purification from *E. coli*.

The expression plasmid TM₄₅₆GGM was transformed into *E. coli*. B834 (DE3) cells. The target protein was expressed with a N-terminal DsbA enzyme fusion to a leader sequence containing hexahistidine tag, S tag and an enterokinase cleavage site. The bacteria were grown at 37 °C in M9AA medium (1 L) supplemented with 1 mM MgSO₄, 0.1 mM CaCl₂, 0.4 % (wt/v) glucose, thiamine chloride (1 mg/L), kanamycin (35 mg/L) and all proteinogenic amino acids. Upon reaching an OD_{600nm} value between 0.8 and 1.0, exchange of medium was performed by centrifugation to remove methionine from the media. The culture was centrifuged for 10 min at 4000 × g at 4°C to remove the supernatant, and the cell pellet was washed three times with 200 mL of M9 medium. The cell 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 (40 mg/L). Protein expression was induced with 0.5 mM IPTG, and the culture was incubated at 25 °C overnight. Cells were harvested by centrifugation for 30 min at 8000 × g at 4 °C. Next, lysis buffer (20 mM Tris-HCl, pH 8.0, and 300 mM NaCl) with the protease inhibitor PMSF (10 µg/mL) was added to a total volume of 50 mL. The cells were then sonicated every 2 min with a probe sonicator (Sonifier 450, Branson Ultrasonics, CT) on ice. Cell disruption was evidenced by partial clearing of the suspension. Then the broken cells were centrifuged 15000 × g for 30 min and the supernatant was collected and directly applied to an immobilized metal affinity column (HisTrap FF) equilibrated with washing buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 20 mM imidazole). The column was washed with 10 column volumes of washing buffer, after which the column was flushed with 3 column volumes of cleavage buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 250 mM imidazole) to afford DsbA-His₆-Stag-rTM₄₅₆-N₃. Finally, enterokinase was used for site-specific cleavage to remove the fusion tags and generate the target protein rTM₄₅₆-N₃, which was purified by passing the reaction mixture through His-trap FF column.

A similar procedure was used for preparing positive control protein DsbA-His₆-Stag-rTM₄₅₆ without incorporation of azide group. Briefly, the expression plasmid TM₄₅₆GGM was transformed into *E. coli*. BL21 (DE3) cells. Bacteria were grown in LB media under the same conditions. rTM₄₅₆ purification and fusion tag3 removal were performed under the same conditions above.

Mass spectrometry analysis of the recombinant TM₄₅₆-N₃

After electrophoresis, the gels immediately was rinsed in dH₂O, and then immersed into Zinc-imidazole staining buffer (Bio-rad). After staining, the gel was rinsed completely in dH₂O. Then protein band was cut from gel and destained according to standard protocols as

manufacture instruction. Next, 3 pieces of gels were crushed and immersed into 1 mL extraction buffer (Formic acid/ water/2-propanol (1:3:2 v/v/v) (FWI) for 2 h, and then were centrifuged at 15,000 rpm for 20 min. The extraction solution was lyophilized and a MALDI matrix solution (formic acid:water:isopropanol (FWI) (1:3:2, v/v/v) saturated with 4-hydroxy- α -cyano-cinnamic acid (4HCCA)) was added to redissolve and prepare the protein for MS analysis using the dried-drop method of matrix crystallization. The MALDI MS experiments were carried out on a Bruker Ultraflex III tandem time-of-flight (TOF/TOF) mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with a Nd:YAG laser emitting at a wavelength of 355 nm. All spectra were measured in positive reflector mode. The instrument was calibrated externally with a poly(methyl methacrylate) standard prior to each measurement. Data analysis was conducted with the flexAnalysis software.

Preparation of TP-PEG functionalized liposome

Lipids mixture of DSPC (15 mg, 20.43 μ mol) cholesterol (4 mg, 10.20 μ mol), mPEG₂₀₀₀-DSPE (4.6 mg, 1.67 μ mol), TP-PEG₃₄₀₀-DSPE (1.5 mg, 0.33 μ mol) (2:1:5%:1% molar ratio) were first dissolved in chloroform (3 mL). The solvent was gently removed on an evaporator under reduced pressure to form a thin lipids film on the flask wall and kept in a vacuum chamber overnight. Then, the lipids film was swelled in the dark with 2.5 mL Tris-HCl buffer (pH 7.4), followed by 10 freeze-thaw cycles of quenching in liquid N₂ and then immersed in a 50 °C water-bath to form multilamellar vesicle suspension. Finally, the crude lipids suspension was extruded through polycarbonate membranes (pore size 600, 200, and 100 nm, gradually) at a 60 °C to afford small unilamellar vesicles. As in the same procedure, a liposome without TP-PEG₃₄₀₀-DSPE was prepared as control. Dynamic light scattering (DLS) was used to monitor the integrity of the vesicles.

Synthesis of rTM₄₅₆-liposome conjugates *via* Staudinger ligation

rTM₄₅₆-N₃ (200 μ g) was purified on a His-trap FF column and transferred into 50 μ L of Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4). The solution was added into 2 mL of TP-PEG-functionalized liposome (TP-PEG₃₄₀₀-DSPE, 1%) described above. The conjugation was then conducted at room temperature for up to 12 h with very gentle shaking under N₂ atmosphere. The un-reacted rTM₄₅₆-N₃ was removed by gel filtration (1.5 $\times$ 20 cm column of Sephadex G-50) to generate rTM₄₅₆-liposome conjugate *via* Staudinger ligation. As in the same procedures above, incubation of rTM₄₅₆ without azide with TP-PEG-functionalized liposome (TP-PEG₂₀₀₀-DSPE, 1%) prepared above in Tris-HCl buffer was investigated as control. DLS was used to monitor the integrity of the vesicles.

Fluorescent dye modification of rTM₄₅₆-N₃ *via* copper-free click chemistry

DsbA-His₆-Stag-rTM₄₅₆-N₃ (200 μ g) and rTM₄₅₆-N₃ (200 μ g) were incubated with cyclooctyne functionalized Fluorescent dye Alexa Fluor® 647 DIBO (70 μ g and 180 μ g, 10 equiv, Invitrogen) in a Tris-HCl buffer overnight at room temperature, respectively. DsbA-His₆-Stag-rTM₄₅₆ without azide was used as a negative control and subjected to the same reaction condition. Protein samples were dialyzed against the buffer to remove un-reacted fluorescent dye and then analyzed by SDS-PAGE. The gel was stained with Coomassie blue and the fluorescent image from the gel was detected using an imaging system (Typhoon 9410 Variable Mode Imager, Amersham Biosciences, USA).

Preparation of DBCO-functionalized liposomes

DSPC (15 mg, 20.43 μ mol), cholesterol (4 mg, 10.2 μ mol), mPEG₂₀₀₀-DSPE (4.6 mg, 1.67 μ mol), DBCO-PEG₂₀₀₀-DSPE (2.2 mg, 0.65 μ mol) (2:1:5%:2% molar ratio) 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 an evaporator under reduced pressure followed

by placing the vessel under vacuum overnight to form a dry thin lipids film on the flask wall. Then the lipids film was swelled with 2.5 mL Tris-HCl buffer to form a multilamellar vesicle suspension. Ten freeze-thaw cycles using liquid N₂ followed by immersion in a 60 °C water bath were performed. The crude lipids suspension was extruded through polycarbonate membranes (millipore size from 600 nm, 200 nm and 100 nm, successively) to produce small unilamellar vesicles. DLS was used to monitor the integrity of the vesicles.

Synthesis of rTM₄₅₆-liposome conjugate *via* copper-free click chemistry

DsbA-His₆-Stag-rTM₄₅₆-N₃ (200 µg) was purified on a His-trap FF column and transferred into 50 µL of Tris-HCl buffer. The solution was added into 2 mL of DBCO-functionalized liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) described above. The click conjugation was then conducted at room temperature for up to 9 h with very gentle shaking. The un-reacted DsbA-His₆-Stag-rTM₄₅₆-N₃ was removed by gel filtration (1.5 × 20 cm column of Sephadex G-50) to afford DsbA-His₆-Stag-rTM₄₅₆ liposome conjugate *via* copper-free click chemistry. As in the same procedures above, the targeted rTM₄₅₆-liposome conjugate was synthesized by releasing the DsbA-His₆-Stag of DsbA-His₆-Stag-rTM₄₅₆-N₃ (200 µg) and followed by incubation with 2 mL of DBCO-functionalized liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) prepared above in Tris-HCl buffer. Also, as in the same procedures above, incubation of rTM₄₅₆ without azide with DBCO-PEG-functionalized liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) prepared above in Tris-HCl buffer was investigated as control experiment. DLS was used to monitor the integrity of the vesicles during and after the coupling reaction.

Bradford assay to determine grafted rTM₄₅₆ amount in rTM₄₅₆-liposome conjugates

1 mL of purified rTM₄₅₆-liposome conjugate from CL 6B column was collected in an acetone compatible tube, followed by adding 4 mL cool acetone (-20 °C). The mixture was vortexed and incubated for 60 min at -20 °C, followed by centrifugation for 10 min at 15,000 × g. Then, supernatant was removed carefully without dislodging the protein pellet. Next, the acetone was allowed to evaporate from the uncapped tube at room temperature for 30 min. 1 mL of Tris-HCl buffer was added for the downstream process and vortexed thoroughly to dissolve protein pellet. Finally, the protein assay was performed as instruction manual of Bradford protein assay (Bio-rad).

Evaluation stability of liposome during copper-free click conjugation and stability of the liposomal rTM₄₅₆ conjugate

The stability of the liposomes during coupling reaction and the final liposomal rTM₄₅₆ conjugate products were monitored by measuring fluorescent leakage in comparison to the same type of liposomes unmodified and having encapsulated self-quenching concentrations of 5,6-carboxyfluorescein (85 mM) using FluoroMax-2 (ISA, NJ). Briefly, the lipids film composed of DSPC, cholesterol, mPEG₂₀₀₀-DSPE, DBCO-PEG₂₀₀₀-DSPE (2:1:5%:2% molar ratio) was swelled in the dark with 2.5 mL Tris-HCl buffer containing 85 mM 5,6-Carboxyfluorescein (5,6-CF) to form the multilamellar vesicle suspension. The crude lipids suspension was extruded through polycarbonate membranes (millipore sizes from 600 nm, 200 nm and 100 nm, successively) to produce small unilamellar vesicles with an average mean diameter of 120 ± 10 nm, as judged by DLS. Separation of the CF vesicles from non-entrapped CF was achieved by gel filtration chromatography, which involved passage through a 1.5 × 20 cm column of Sephadex G-50. Copper free click conjugation was conducted using the same procedure above. 20 µL of reaction solution was taken and mixed with 1980 µL of Tris-HCl buffer, and then the fluorescent intensity was measured by using FluoroMax-2. A control experiment was conducted, in which recombinant TM₄₅₆ without azide group was used in equal amount. To evaluate extent of fluorescence release upon disruption in the conjugation, 20 µL of 0.5 % Triton-100 Tris-HCl buffer solution was

added into sample above to release all entrapped CF molecules from the liposomes and fluorescence intensity was measured.

Protein C activation assay of DsbA-His₆-Stag-rTM₄₅₆-N₃, rTM₄₅₆-N₃ and liposomal rTM₄₅₆ conjugates

The activity of recombinant TM₄₅₆ was defined as moles of produced active Protein C per min by given amounts of rTM₄₅₆ and rTM₄₅₆ conjugates in the presence of thrombin. All activations of protein C by rTM₄₅₆ and rTM₄₅₆ conjugates were performed for 60 min in 20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37°C. Typically, 20 nM thrombin and different rTM₄₅₆ concentrations (1, 1.5, and 2 nM) were incubated in the assay buffer. After 60 min, 20 μL antithrombin III (3 μg/μL) and 20 μL heparin (1000 IU/mL) were added into the solution to stop the reaction. The inhibition of protein C activation was completed within 5 min at room temperature. The produced activated PC was measured through hydrolysis of a chromogenic substrate (Spectrozyme PCa) by comparing a standard curve, in which the concentration of active protein C to the rate of *p*-nitroanilide (*p*NA) formation was measured. The hydrolysis of Spectrozyme PCa was performed for 10 min in the assay buffer at 37 °C, in which *p*NA was produced and its concentration measured by monitoring at λ = 405 nm with a UV spectrophotometer.

RESULTS AND DISCUSSION

Synthesis of rTM₄₅₆-Azide

A truncated TM fragment containing EGF4-6 with the insertion of a C-terminal nonnatural methionine analogue (rTM₄₅₆-N₃) was chosen as our target antithrombotic protein for site-specific conjugation. Specifically, a truncated recombinant TM mutant containing amino acid sequences 349-492 with a Met-388-Leu substitution and a C-terminal linker GlyGlyMet-N₃ was constructed using site-directed mutagenesis.²⁰ In this study, recombinant TM₄₅₆ with DsbA, His-tag and S-tag at the N-terminal and an azidohomolanine at the C-terminal was expressed in *E. coli*.²⁷ The target recombinant TM₄₅₆ fused to the leader sequence was clearly detected in the positive control cultures supplemented with azidohomoalanine, whereas it was not observed in the negative control culture. The DsbA-His₆-Stag-rTM₄₅₆-N₃ was purified from the cell pellet by using metal-affinity chromatography with stepwise imidazole-gradient elution under native conditions. Enterokinase cleavage removed the fusion tag and generated the target rTM₄₅₆-N₃. N-terminal sequencing, amino acid composition confirmed the protein integrity. The rTM₄₅₆-N₃ was characterized by SDS-PAGE gel (Figure 2A) and mass spectrometry analysis (M_w = 16645 D, Figure 2B).

Synthesis of rTM₄₅₆-liposome conjugate via Staudinger ligation

Conventional methods in the preparation of surface functionalized liposomes involve the initial synthesis of the key lipid-ligand conjugate, followed by formulation of the liposome with other lipid components. In this direct liposome formulation method, some of the valuable ligands inevitably face the enclosed aqueous compartment and thus become unavailable for their intended interaction with their target molecules. Particularly, in a liposome formulation for drug delivery, it is unrealistic if the targeting ligand is only available in minimal amounts, which may hinder the ability of the carrier to reach its destination and to properly interact with its target especially when multivalent binding is necessary. In addition, lipid-ligand conjugates may have poor solubility and stability in aqueous solvent, or are incompatible with liposome preparation processes. In the present study, the chemically selective post-modification approach was investigated to generate the proposed rTM₄₅₆-liposome conjugate by taking the advantage of the efficient utilization of rTM₄₅₆ on the outer leaflet and avoiding solubility problems in the formation of a lipid-

protein conjugate as well. Briefly, liposome incorporation of anchor lipid TP-PEG₃₄₀₀-DSPE for Staudinger ligation was prepared. Then, rTM₄₅₆-N₃ was incubated with the liposome at room temperature for 12 h and followed by purification through CL-6B column chromatography to afford the liposomal rTM₄₅₆ conjugate. Control experiments between rTM₄₅₆-N₃ and liposome without anchor lipid and between rTM₄₅₆ and liposome with anchor lipid were conducted in the same condition. As shown in Figure 3, the reaction between rTM₄₅₆-N₃ and liposome with anchor lipid was observed as a new band formation (Figure 3A), and the conjugation reached maximum yield at 12 h. On the other hand, the control experiment between rTM₄₅₆-N₃ and liposome without anchor lipid (Figure 3B) and between rTM₄₅₆ and liposome with anchor lipid (Figure 3C) did not exhibit any new band formed. These results indicated successful liposome modification of rTM-N₃ *via* Staudinger ligation.

The conjugation efficiency of the Staudinger ligation conjugation was evaluated by a given amount of rTM₄₅₆-N₃ and fixed anchor lipid (TP-PEG₃₄₀₀-DSPE) concentration in the liposome (1 mol % of the lipids in liposome), in which TP-PEG₃₄₀₀-DSPE was in excess to rTM₄₅₆-N₃. The grafted amount of rTM₄₅₆-N₃ onto the liposome was calculated after gel filtration purification (CL 6B, GE Healthcare) by measuring the rTM₄₅₆-PEG₃₄₀₀-DSPE conjugate formed and the un-reacted rTM₄₅₆-N₃ in the buffer, respectively. Protein precipitation with acetone was used to separate protein pellet from the liposomal solution. The pellet was then redissolved in Tris-HCl buffer (pH 8.0) and measured by the Bradford protein assay (Bio-rad). As a result, the conjugation yield was 21% by measuring the rTM₄₅₆-liposome conjugate formed and 30% by measuring the un-reacted rTM₄₅₆-N₃ left, respectively. The gap between the calculated values of direct and indirect method might come from loss of un-reacted rTM₄₅₆-N₃ during the purification process. The disadvantage of Staudinger ligation is the oxidation of the triphenylphosphine to triphenylphosphate during sample preparation and conjugation reaction.³⁹ This might be the main reason of the low conjugation yield of Staudinger ligation.

Synthesis of rTM₄₅₆-liposome conjugate *via* copper-free click chemistry

Recently, copper-free click chemistry has emerged as a popular bioorthogonal ligation strategy for biomolecule modification.³⁴ In this study, we explored regio- and chemoselective liposomal functionalization of the rTM₄₅₆-N₃ at the C-terminus through copper-free click chemistry. First, to investigate whether the azide moiety in the protein was reactive under copper-free click chemistry condition, both DsbA-His₆-Stag-rTM₄₅₆-N₃ and rTM₄₅₆-N₃ were incubated with cyclooctyne functionalized Fluorescent dye Alexa Fluor® 647 DIBO (10 equiv, Invitrogen) in a Tris-HCl buffer at room temperature. The DsbA-His₆-Stag-rTM₄₅₆ without azide was used as a negative control and subjected to the same reaction condition. Protein samples were dialyzed against the buffer to remove un-reacted fluorescent dye and then were analyzed by SDS-PAGE. The gel was stained with Coomassie blue (Figure 4A) and the fluorescent image from the gel were detected using an imaging system (Typhoon 9410 Variable Mode Imager, Amersham Biosciences, USA) (Figure 4B). As a result, both DsbA His₆-Stag-rTM₄₅₆-N₃ and rTM₄₅₆-N₃ were fluorescent, whereas no fluorescent image was detected from the DsbA-His₆-Stag-rTM₄₅₆ without azide group. These results indicated that the azide moiety in the protein was reactive toward the cyclooctyne and could be conjugated to cyclooctyne-containing molecules under copper-free click chemistry condition.

Copper-free click chemistry has been used as a versatile chemistry for biomolecule liposome modification.³⁶ Recently, we have developed liposome surface glyco-functionalization *via* copper-free click chemistry (see Supporting Information). In this study, we investigated the selective liposomal modification of rTM₄₅₆-N₃ *via* copper-free click chemistry. Briefly, liposomes bearing the anchor lipid DBCO-PEG₂₀₀₀-DSPE were prepared as above, followed

by incubation with DsbA-His₆-Stag-rTM₄₅₆-N₃ in a Tris-HCl buffer. The click conjugation was conducted at room temperature for up to 9 h under very gentle shaking. The un-reacted DsbA-His₆-Stag-rTM₄₅₆-N₃ was then removed by gel filtration (1.5 × 20 cm column of Sephadex G-50) to afford the targeted DsbA-His₆-Stag-rTM₄₅₆-liposome conjugate. Western blotting confirmed the new band formed upon DsbA-His₆-Stag-rTM₄₅₆-N₃ reacted with the liposome bearing the anchor lipid DBCO-PEG₂₀₀₀-DSPE (Figure 5A). As in the same procedure, the targeted rTM₄₅₆-liposome conjugate was synthesized by reacting the rTM₄₅₆-N₃ with liposomes bearing the anchor lipid DBCO-PEG₂₀₀₀-DSPE in Tris-HCl buffer. As shown in Figure 5B, Western blotting confirmed the new band formed upon the rTM₄₅₆-N₃ reacted with the liposome. Both reactions were monitored for 9 hrs, which indicated that no more products formed after that time. On the other hand, the control experiment between rTM₄₅₆ without azide and liposome with anchor lipid did not exhibit any new band formed (Figure 3C). These results indicated successful liposomal modification of the rTM₄₅₆-N₃ *via* copper-free click chemistry.

The conjugation efficiency of the copper-free click conjugation was evaluated by a given amount of rTM₄₅₆-N₃ and fixed anchor lipid (DBCO-PEG₂₀₀₀-DSPE) concentration in the liposome (2% of the lipids in liposome). The grafted amount of rTM₄₅₆-N₃ onto the liposome was determined after gel filtration purification (CL 6B, GE Healthcare) by measuring rTM₄₅₆-PEG₂₀₀₀-DSPE formed and unreacted rTM₄₅₆-N₃ in the buffer, respectively, in which DBCO-PEG₂₀₀₀-DSPE was in excess to rTM₄₅₆-N₃. Acetone proteins precipitation was used for the physical separation of protein pellet from the liposomal solution. The pellet was then redissolved in Tris-HCl buffer and detected by the Bradford protein assay (Bio-rad). As a result, the conjugation yield was ~60% by measuring the rTM₄₅₆ liposome conjugate formed and ~68% by measuring the un-reacted rTM₄₅₆-N₃, respectively. Apparently, copper-free click chemistry afforded higher yield than Staudinger ligation above. DLS was used to monitor the liposomal conjugation of the rTM₄₅₆-N₃ *via* copper-free click conjugation and to verify the integrity of the liposome during and after the click conjugation reaction. As shown in Figure 6, the average of liposome size increased from 118 ± 5 nm (Figure 6A) to 140 ± 5 nm (Figure 6B). After separation of the rTM₄₅₆-liposome conjugate from the reaction solution, the rTM₄₅₆-liposome conjugate did not show size change (Figure 6C). Furthermore, there was no size change of control experiment (119 ± 10 nm) in the presence of rTM₄₅₆ without azide group (Figure 6D). These results indicated that the liposomes were intact during the reaction and purification process.

To test whether the copper-free click conjugation reaction condition could affect the integrity of the liposome and the stability of liposomal conjugate as well, fluorescent dye leakage of the same type of liposomes having encapsulated self-quenching concentration of 5,6-CF (85 mM) was monitored using FluoroMax-2 (ISA) spectrometer. Based on the fluorescence quenching and releasing assay of rTM₄₅₆-liposome conjugate (Figure 7A), no apparent leakage was triggered by the conjugation reaction compared with the control liposomes incubated in the presence of rTM₄₅₆ without azide group. This fluorescence intensity unchanging indicated that no liposome disruption occurred during the copper-free click conjugation reaction. In addition, the conjugation of the rTM₄₅₆-N₃ to the liposome surface may have enhanced the liposome stability as it exhibited significantly slower dye release between 14 to 21 days as compared to the control liposome treated with rTM₄₅₆ without azide group (Figure 7B). These results indicated the compatibility of the copper-free click chemistry for rTM₄₅₆-liposome formation, and the rTM₄₅₆-liposome conjugate was quite stable and could be used for further activity evaluation.

Protein C activation activity of rTM₄₅₆-N₃ and its liposomal conjugates

In this study, protein C activation activities of rTM₄₅₆-N₃ and its liposomal conjugates were evaluated. The activity of rTM₄₅₆ was defined as moles of produced activated protein C per

min by given amounts of rTM₄₅₆-N₃ and rTM₄₅₆ conjugates in the presence of thrombin and calcium. All protein C activation assays by rTM₄₅₆-N₃ and rTM₄₅₆-liposome conjugates were performed for 60 min in a buffer with 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca²⁺ at 37 °C, as previously reported.⁴⁰ As shown in Table 1, rTM₄₅₆-liposome conjugate made *via* either Staudinger ligation or copper-free click chemistry showed a 2-fold higher $k_{\text{cat}}/K_{\text{m}}$ value than that of either DsbA-His₆-Stag-TM₄₅₆-N₃ or rTM₄₅₆-N₃, even though there was no apparent change of k_{cat} . This change in $k_{\text{cat}}/K_{\text{m}}$ mainly was due to a decreased K_{m} value, which represents the affinity of rTM₄₅₆ for protein C. This result was consistent with a previous report of increased $k_{\text{cat}}/K_{\text{m}}$ values due to a higher affinity of rTM₄₅₆ for protein C. It has been known that anticoagulant reactions occur at a physiologically significant rate only when the respective enzymes form multicomponent complexes on lipid membrane surfaces.⁴¹ Esmon, *et al.* had demonstrated that TM incorporated into lipid vesicles resulted in substantially enhanced protein C activation.⁴² Therefore, our result indicated that the increased activity may result from using liposome as a platform, which mimics endothelial cell lipid membrane and has a beneficiary effect for enhancing thrombin and protein C binding. In addition, the dense rTM on the liposome surface may also contribute to its enhanced activity.

CONCLUSION

We demonstrated here the first example of bio-inspired liposomal TM conjugates that were synthesized by site-specific conjugation of recombinant TM₄₅₆-N₃ at the C-terminus with liposome *via* Staudinger ligation and copper-free click chemistry, respectively. We found that copper-free click chemistry afforded higher yield than Staudinger ligation for liposomal protein conjugation. The protein conjugation method presented here has distinct advantages over traditional methods. First, the site-specific, chemo- and bio-orthogonal conjugation provides a simple and convenient route to uniform protein conjugation in good yields. Second, mild conjugation condition minimizes the chance of protein denaturation. The rTM₄₅₆-liposome conjugate showed a 2-fold higher $k_{\text{cat}}/K_{\text{m}}$ value for protein C activation than that of rTM₄₅₆ alone, which indicated that the lipid membrane has a beneficiary effect on the rTM₄₅₆'s activity. Continued studies of *in vitro* and *in vivo* antithrombotic activity of these TM conjugates and their pharmacokinetic properties are under investigation. Overall, the proposed membrane-mimetic re-expression of recombinant TM₄₅₆ onto liposome provides a rational design strategy for facilitating studies of membrane protein TM functions and is expected to generate a potential TM-based antithrombotic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic illustration of synthesis of bio-inspired liposomal rTM₄₅₆ conjugates *via* recombinant and bio-orthogonal chemistry strategy. D1-D6 are six domains of TM, 1-6 are six epidermal growth factor-like (EGF) domains in the D2 domain, rTM₄₅₆ is recombinant TM containing EGF-like domains 4, 5 and 6.

Figure 2. SDS-PAGE (4-20%) characterization of recombinant DsbA-His₆-Stag-rTM₄₅₆-N₃ (A) and MALDI mass characterization of purified recombinant TM₄₅₆-N₃ (Matrix solution: formic acid:water:isopropanol (FWI) (1:3:2, v/v/v) saturated with 4-hydroxy- α -cyano-cinnamic acid (4HCCA)).

Figure 3.

Western blot characterization of site specific liposomal modification of rTM₄₅₆-N₃ with liposome having TP-PEG₃₄₀₀-DSPE (**A**) and with liposome without TP-PEG₃₄₀₀-DSPE (**B**) and of rTM₄₅₆ without N₃ with liposome having TP-PEG₃₄₀₀-DSPE (**C**) *via* Staudinger ligation. The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA) was used.

Figure 4. SDS-PAGE (12%) characterization of recombinant DsbA-His₆-Stag-rTM₄₅₆, DsbA-His₆-Stag-rTM₄₅₆-N₃ and recombinant TM₄₅₆-N₃ upon copper-free click chemistry modification with cyclooctyne functionalized Fluorescent dye Alexa Fluor® 647 DIBO: **A**. Coomassie blue staining, **B**. Fluorescence scanning. Tags-rTM₄₅₆: DsbA-His₆-Stag-rTM₄₅₆.

Figure 5.

Western blot characterization of site specific liposomal modification of DsbA-His₆-Stag-rTM₄₅₆-N₃ (A) and of rTM₄₅₆-N₃ (B) and of rTM₄₅₆ (C) with liposome carrying anchor lipid DBCO-PEG₂₀₀₀-DSPE *via* copper free click chemistry. The mouse monoclonal antibody specific to human TM was from COVANCE Corp. (Richmond, CA) was used. Tags-rTM₄₅₆: DsbA-His₆-Stag-rTM₄₅₆.

Figure 6.

DLS monitoring of liposome stability during copper-free click chemistry conjugation between liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) and rTM₄₅₆-N₃: **A.** before conjugation; **B.** after conjugation; **C.** purified rTM₄₅₆-liposome conjugate; **D.** control: liposome (DBCO-PEG₂₀₀₀-DSPE, 2%) treated with rTM₄₅₆ (without N₃).

Figure 7.

Evaluation of stability of liposome during copper-free click conjugation reaction (**A**) and stability of rTM₄₅₆-liposome conjugate (**B**) by monitoring fluorescent dye 5,6-CF releasing from liposomes. Disruption of liposomes was conducted by adding PBS buffer containing 0.5% Triton X-100 surfactant into the liposome solutions at the final point. Total is the total amount of 5,6-CF determined after disruption of liposome.

Table 1

Protein C activation activity of rTM456-N₃ and its conjugates

	Full TM ^{*1}	rTM456-N ₃ ^{*1}	Tags-rTM456-N ₃	rTM456-N ₃	rTM456-Liposome (SL)	rTM456-Liposome (CFCC)
<i>K_m</i> (μM)	0.60±0.15	1.00±0.5	0.80±0.2	0.90±0.2	0.31±0.15	0.33±0.17
<i>k_{cat}</i> (min ⁻¹)	0.20±0.03	0.16±0.05	0.28±0.05	0.24±0.04	0.24±0.07	0.23±0.17
<i>k_{cat}/K_m</i> (min ⁻¹ ·μM ⁻¹)	0.37±0.14	0.16±0.05	0.40±0.15	0.29±0.11	0.77±0.26	0.71±0.32

^{*1} Commercial human full TM

^{*2} Protein C activation activity of rTM456-N₃ reported by Cazalis et al.²⁰, Tags-rTM456-N₃: DsbA-His6-Stag-rTM456-N₃, SL: Staudinger ligation, CFCC: copper-free click chemistry