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Bio-Inspired Liposomal Thrombomodulin Conjugate through Bio-Orthogonal Chemistry

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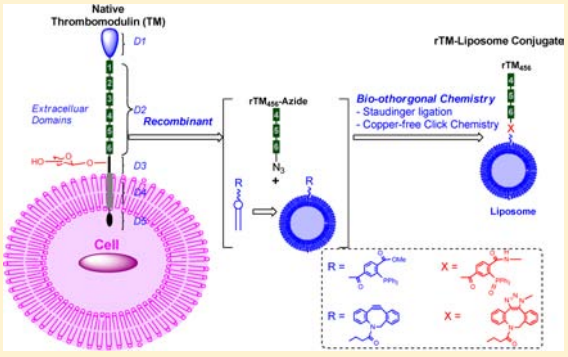
Supporting Information

ABSTRACT: We report the synthesis of bioinspired 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 bioinspired 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 rational 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 (rhsTMα or ART-123), which was recently approved in Japan to treat patients with disseminated intravascular coagulation (DIC).[13,14]

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 (rTM_{456}) has been considered to be a sole anticoagulant candidate.[17−20] However, rTM_{456} has a very short half-life (6−9 min) in animals, as compared to that of 5 h for rhsTM.[21] Therefore, the pharmacokinetic properties of rTM_{456}, 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,

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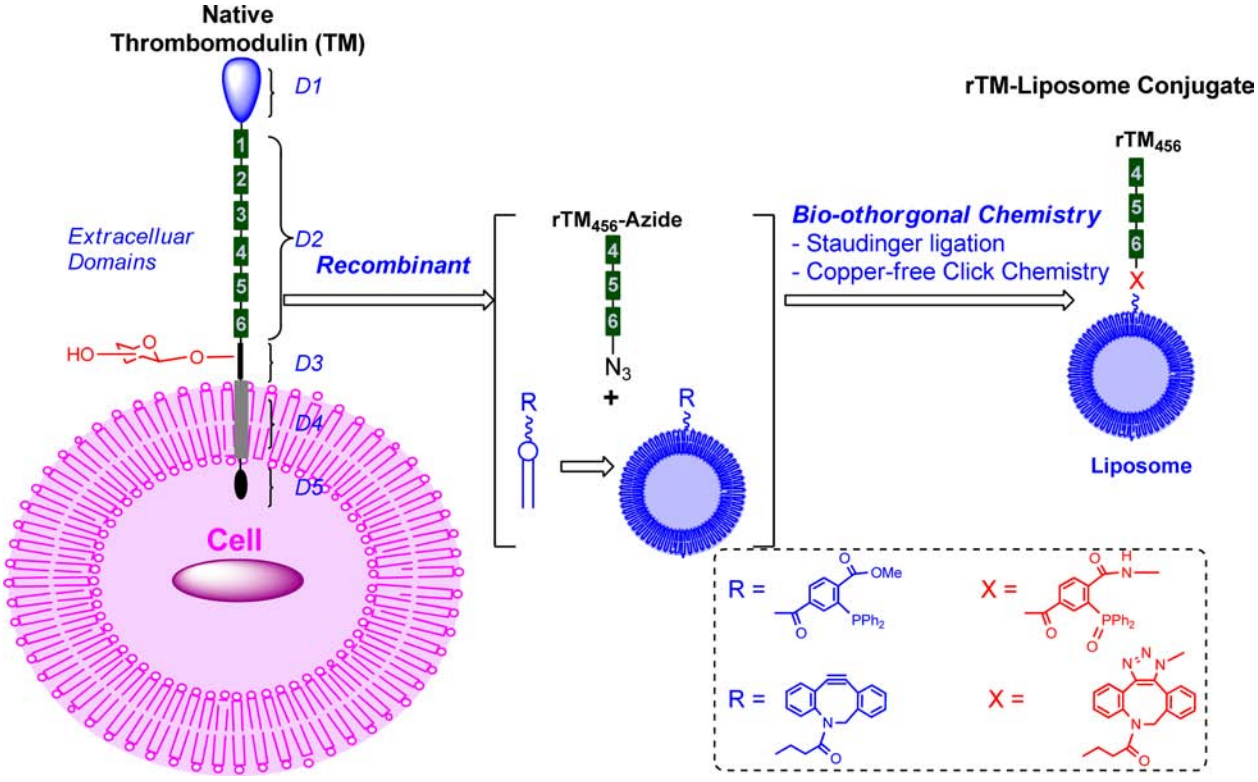

Figure 1. Schematic illustration of synthesis of bioinspired liposomal rTM_{456} conjugates via recombinant and bio-orthogonal chemistry strategy. D1−D6 are six domains of TM; 1−6 are six epidermal growth factor-like (EGF) domains in the D2 domain; rTM_{456} is recombinant TM containing EGF-like domains 4, 5, and 6.

enzymes, drugs, and genes to sites of action.[23] In this study, we envisioned that liposomal rTM conjugates containing rTM_{456} 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 chemistry,[29] 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 liposome[36] 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 TM_{456} derivative at the C-terminus through Staudinger ligation and copper-free click chemistry, respectively. The bioinspired 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, *Bam*HI-HF, *Kpn*I-HF, T4 DNA ligase, and alkaline phosphatase

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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 antithrombin, 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-Disteroyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-disteroyl-*sn*-glycero-3-phosphoethanol-amine-*N*-[amino(polyethylene glycol)2000] (NH_2−PEG_{2000}−DSPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) were from Avanti Polar Lipids (Alabaster, AL). DBCO-PEG_4-NHS ester was from Click Chemistry Tools (Scottsdale, AZ). Cholesterol, imidazole, sodium azide, *N*,*N*-dimethylformamide, and other chemicals were purchased from Sigma-Aldrich (USA). Triphenylphosphine (TP)-PEG_{3400}-DSPE was synthesized as described previously.[38]

Synthesis of DBCO-PEG_{2000}-DSPE. To a dry, 25 mL round-bottom flask containing a stir a bar, DSPE-PEG_{2000}-NH_2 (94.4 mg, 0.034 mmol) was added and the flask was then equipped with a three-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-PEG_4-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:$CHCl_3$ 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-PEG_{2000}-DSPE (77 mg, 79% yield). ^{1}H NMR ($CDCl_3$, 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). ^{13}C NMR ($CDCl_3$, 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 TM_{456} was prepared by expressing plasmid TM_{456}-N_3 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 TM_{456}GGM. The sequence of each mutant plasmid was confirmed by DNA sequencing.

Mutant TM_{456} Expression and Purification from *E. coli*. The expression plasmid TM_{456}GGM 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 $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% (wt/v) glucose, thiamine chloride (1 mg/L), kanamycin (35 mg/L), and all proteinogenic amino acids. Upon reaching an $OD_{600\ nm}$ 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 15 000*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-His_6-Stag-rTM_{456}-N_3. Finally, enterokinase was used for site-specific cleavage to remove the fusion tags and generate the target protein rTM_{456}-N_3, which was purified by passing the reaction mixture through His-trap FF column.

A similar procedure was used for preparing positive control protein DsbA-His_6-Stag-rTM_{456} without incorporation of azide group. Briefly, the expression plasmid TM_{456}GGM was transformed into *E. coli* BL21 (DE3) cells. Bacteria were grown in LB media under the same conditions. rTM_{456} purification and fusion tag3 removal were performed under the same conditions above.

Mass Spectrometry Analysis of the Recombinant TM_{456}-N_3. After electrophoresis, the gels immediately was rinsed in dH_2O, and then immersed into zinc-imidazole staining buffer (Biorad). After staining, the gel was rinsed completely in dH_2O. Then, protein band was cut from gel and destained according to standard protocols per manufacturer's instructions. 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-

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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 μmol) cholesterol (4 mg, 10.20 μmol), mPEG$_{2000}$-DSPE (4.6 mg, 1.67 μmol), TP-PEG$_{3400}$-DSPE (1.5 mg, 0.33 μmol) (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 lipid film on the flask wall and kept in a vacuum chamber overnight. Then, the lipid 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 N_2, and then immersed in a 50 °C water bath to form multilamellar vesicle suspension. Finally, the crude lipid suspension was extruded through polycarbonate membranes (pore size 600, 200, and 100 nm, gradually) at a 60 °C to afford small unilamelar vesicles. As in the same procedure, a liposome without TP-PEG$_{3400}$-DSPE was prepared as control. Dynamic light scattering (DLS) was used to monitor the integrity of the vesicles.

Synthesis of rTM$_{456}$-Liposome Conjugates via Staudinger Ligation. rTM$_{456}$-N$_3$ (200 μg) was purified on a His-trap FF column and transferred into 50 μL 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-PEG$_{3400}$-DSPE, 1%) described above. The conjugation was then conducted at room temperature for up to 12 h with very gentle shaking under N_2 atmosphere. The unreacted rTM$_{456}$-N$_3$ was removed by gel filtration (1.5 × 20 cm column of Sephadex G-50) to generate rTM$_{456}$-liposome conjugate via Staudinger ligation. As in the same procedures above, incubation of rTM$_{456}$ without azide with TP-PEG-functionalized liposome (TP-PEG$_{2000}$-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 rTM$_{456}$-N$_3$ via Copper-Free Click Chemistry. DsbA-His$_6$-Stag-rTM$_{456}$-N$_3$ (200 μg) and rTM$_{456}$-N$_3$ (200 μg) were incubated with cyclooctyne functionalized fluorescent dye Alexa Fluor 647 DIBO (70 μg and 180 μg, 10 equiv, Invitrogen) in a Tris-HCl buffer overnight at room temperature, respectively. DsbA-His$_6$-Stag-rTM$_{456}$ without azide was used as a negative control and subjected to the same reaction condition. Protein samples were dialyzed against the buffer to remove unreacted 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 μmol), cholesterol (4 mg, 10.2 μmol), mPEG$_{2000}$-DSPE (4.6 mg, 1.67 μmol), and DBCO-PEG$_{2000}$-DSPE (2.2 mg, 0.65 μmol) (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 lipid 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 N_2 followed by immersion in a 60 °C water bath were performed. The crude lipids suspension was extruded through polycarbonate membranes (Millipore size from 600, 200, and 100 nm, successively) to produce small unilamellar vesicles. DLS was used to monitor the integrity of the vesicles.

Synthesis of rTM$_{456}$-Liposome Conjugate via Copper-Free Click Chemistry. DsbA-His$_6$-Stag-rTM$_{456}$-N$_3$ (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-PEG$_{2000}$-DSPE, 2%) described above. The click conjugation was then conducted at room temperature for up to 9 h with very gentle shaking. The unreacted DsbA-His$_6$-Stag-rTM$_{456}$-N$_3$ was removed by gel filtration (1.5 × 20 cm column of Sephadex G-50) to afford DsbA-His$_6$-Stag-rTM$_{456}$-liposome conjugate via copper-free click chemistry. As in the same procedures above, the targeted rTM$_{456}$-liposome conjugate was synthesized by releasing the DsbA-His$_6$-Stag of DsbA-His$_6$-Stag-rTM$_{456}$-N$_3$ (200 μg) and followed by incubation with 2 mL of DBCO-functionalized liposome (DBCO-PEG$_{2000}$-DSPE, 2%) prepared above in Tris-HCl buffer. Also, as in the same procedures above, incubation of rTM$_{456}$ without azide with DBCO-PEG-functionalized liposome (DBCO-PEG$_{2000}$-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 rTM$_{456}$ Amount in rTM$_{456}$-Liposome Conjugates. One milliliter of purified rTM$_{456}$-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. One milliliter of Tris-HCl buffer was added for the downstream process and vortexed thoroughly to dissolve protein pellet. Finally, the protein assay was performed as an instruction manual of Bradford protein assay (Bio-Rad).

Evaluation Stability of Liposome during Copper-Free Click Conjugation and Stability of the Liposomal rTM$_{456}$ Conjugate. The stability of the liposomes during coupling reaction and the final liposomal rTM$_{456}$ 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, mPEG$_{2000}$-DSPE, and DBCO-PEG$_{2000}$-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, 200, 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 nonentrapped 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

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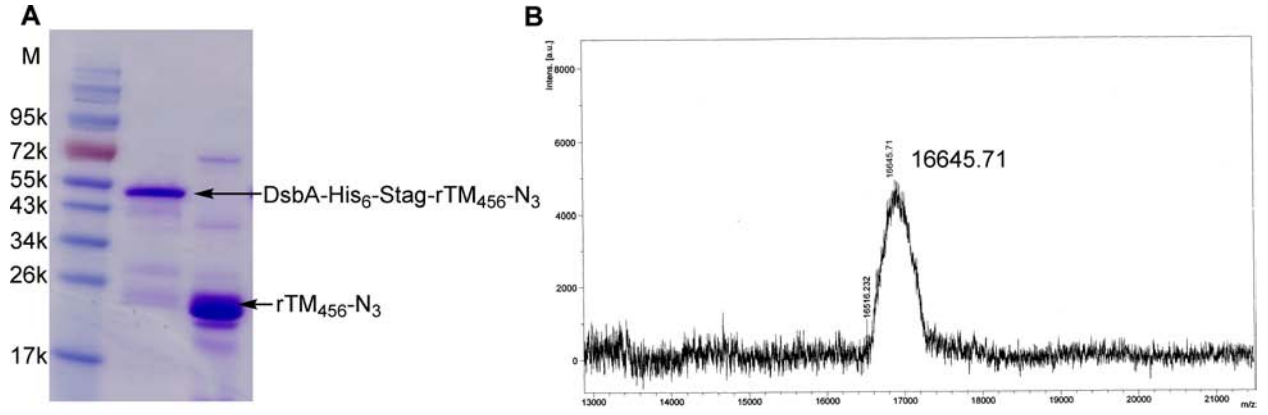

Figure 2. SDS-PAGE (4–20%) characterization of recombinant DsbA-His_6-Stag-rTM_{456}-N_3 (A) and MALDI mass characterization of purified recombinant TM_{456}-N_3 (matrix solution: formic acid:water:isopropanol (FWI) (1:3:2, v/v/v) saturated with 4-hydroxy-α-cyano-cinnamic acid (4HCCA)).

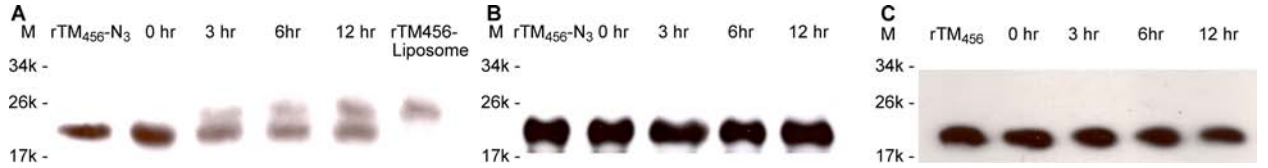

Figure 3. Western blot characterization of site specific liposomal modification of rTM_{456}-N_3 with liposome having TP-PEG_{3400}-DSPE (A) and with liposome without TP-PEG_{3400}-DSPE (B) and of rTM_{456} without N_3 with liposome having TP-PEG_{3400}-DSPE (C) via Staudinger ligation. The mouse monoclonal antibody specific to human TM from COVANCE Corp. (Richmond, CA) was used.

using the same procedure above. Twenty microliters 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 TM_{456} 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 the sample above to release all entrapped CF molecules from the liposomes, and fluorescence intensity was measured.

Protein C Activation Assay of DsbA-His_6-Stag-rTM_{456}-N_3, rTM_{456}-N_3, and Liposomal rTM_{456} Conjugates. The activity of recombinant TM_{456} was defined as moles of produced active Protein C per min by given amounts of rTM_{456} and rTM_{456} conjugates in the presence of thrombin. All activations of protein C by rTM_{456} and rTM_{456} conjugates were performed for 60 min in 20 mM Tris-HCl buffer, pH 7.4, 100 mM NaCl, 0.1% BSA, and 5 mM Ca^{2+} at 37 °C. Typically, 20 nM thrombin and different rTM_{456} 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 rTM_{456}-Azide. A truncated TM fragment containing EGF4–6 with the insertion of a C-terminal nonnatural methionine analogue (rTM_{456}-N_3) 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-N_3 was constructed using site-directed mutagenesis.[20] In this study, recombinant TM_{456} with DsbA, His-tag and S-tag at the *N*-terminal and an azidohomolananine at the C-terminal was expressed in *E. coli*.[27] The target recombinant TM_{456} 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-His_6-Stag-rTM_{456}-N_3 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 rTM_{456}-N_3. *N*-Terminal sequencing and amino acid composition confirmed the protein integrity. The rTM_{456}-N_3 was characterized by SDS-PAGE gel (Figure 2A) and mass spectrometry analysis (M_w = 16 645 D, Figure 2B).

Synthesis of rTM_{456}-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

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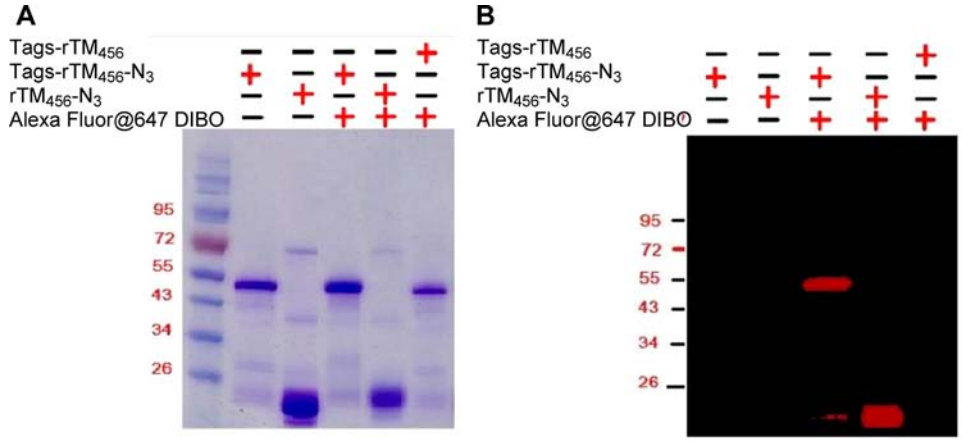

Figure 4. SDS-PAGE (12%) characterization of recombinant DsbA-His_6-Stag-rTM_{456}, DsbA-His_6-Stag-rTM_{456}-N_3, and recombinant TM_{456}-N_3 upon copper-free click chemistry modification with cyclooctyne functionalized fluorescent dye Alexa Fluor 647 DIBO: (A) Coomassie blue staining, (B) fluorescence scanning. Tags-rTM_{456}: DsbA-His_6-Stag-rTM_{456}.

to reach its destination and to properly interact with its target especially when multivalent binding is necessary. In addtion, 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 postmodification approach was investigated to generate the proposed rTM_{456}-liposome conjugate by taking the advantage of the efficient utilization of rTM_{456} 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-PEG_{3400}-DSPE for Staudinger ligation was prepared. Then, rTM_{456}-N_3 was incubated with the liposome at room temperature for 12 h and followed by purification through CL-6B column chromatography to afford the liposomal rTM_{456} conjugate. Control experiments between rTM_{456}-N_3 and liposome without anchor lipid and between rTM_{456} and liposome with anchor lipid were conducted in the same condition. As shown in Figure 3, the reaction between rTM_{456}-N_3 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 rTM_{456}-N_3 and liposome without anchor lipid (Figure 3B) and between rTM_{456} and liposome with anchor lipid (Figure 3C) did not exhibit any new band formed. These results indicated successful liposome modification of rTM-N_3 via Staudinger ligation.

The conjugation efficiency of the Staudinger ligation conjugation was evaluated by a given amount of rTM_{456}-N_3 and fixed anchor lipid (TP-PEG_{3400}-DSPE) concentration in the liposome (1 mol % of the lipids in liposome), in which TP-PEG_{3400}-DSPE was in excess to rTM_{456}-N_3. The grafted amount of rTM_{456}-N_3 onto the liposome was calculated after gel filtration purification (CL 6B, GE Healthcare) by measuring the rTM_{456}-PEG_{3400}-DSPE conjugate formed and the unreacted rTM_{456}-N_3 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 rTM_{456}-liposome conjugate formed and 30% by measuring the unreacted rTM_{456}-N_3 left, respectively. The gap between the calculated values of direct and indirect method might come from the loss of unreacted rTM_{456}-N_3 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 for the low conjugation yield of Staudinger ligation.

Synthesis of rTM_{456}-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 rTM_{456}-N_3 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-His_6-Stag-rTM_{456}-N_3 and rTM_{456}-N_3 were incubated with cyclooctyne functionalized fluorescent dye Alexa Fluor 647 DIBO (10 equiv, Invitrogen) in a Tris-HCl buffer at room temperature. The DsbA-His_6-Stag-rTM_{456} without azide was used as a negative control and subjected to the same reaction condition. Protein samples were dialyzed against the buffer to remove unreacted 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-His_6-Stag-rTM_{456}-N_3 and rTM_{456}-N_3 were fluorescent, whereas no fluorescent image was detected from the DsbA-His_6-Stag-rTM_{456} 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 developed liposome surface glyco-functionalization via copper-free click chemistry (see Supporting Information). In this study, we investigated the selective liposomal modification of rTM_{456}-N_3 via copper-free click chemistry. Briefly, liposomes bearing the anchor lipid DBCO-PEG_{2000}-DSPE were prepared as above, followed by incubation with DsbA-His_6-Stag-rTM_{456}-N_3 in a Tris-HCl buffer. The click conjugation was conducted at room temperature for up to 9 h under very gentle shaking. The unreacted DsbA-His_6-Stag-rTM_{456}-N_3 was then removed by gel filtration (1.5 × 20 cm column of Sephadex G-50) to afford the targeted Dasb-His-Stag-rTM_{456}-liposome conjugate.

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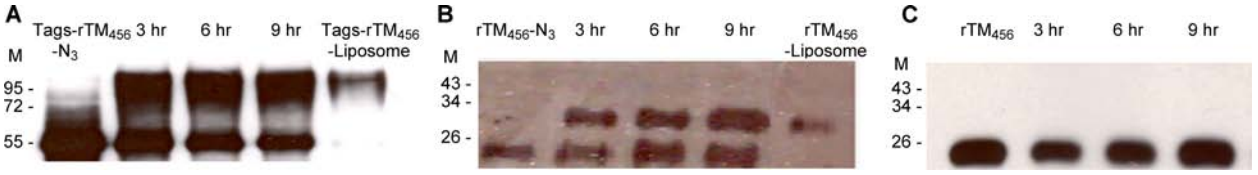

Figure 5. Western blot characterization of site specific liposomal modification of DsbA-His_6-Stag-rTM_{456}-N_3 (A), rTM_{456}-N_3 (B), and rTM_{456} (C) with liposome carrying anchor lipid DBCO-PEG_{2000}-DSPE via copper free click chemistry. The mouse monoclonal antibody specific to human TM from COVANCE Corp. (Richmond, CA) was used. Tags-rTM_{456}: DsbA-His_6-Stag-rTM_{456}.

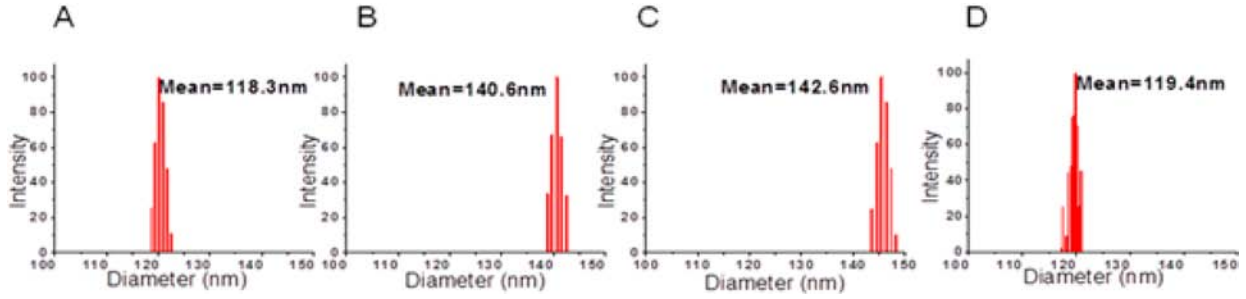

Figure 6. DLS monitoring of liposome stability during copper-free click chemistry conjugation between liposome (DBCO-PEG_{2000}-DSPE, 2%) and rTM_{456}-N_3: (A) before conjugation; (B) after conjugation; (C) purified rTM_{456}-liposome conjugate; (D) control: liposome (DBCO-PEG_{2000}-DSPE, 2%) treated with rTM_{456} (without N_3).

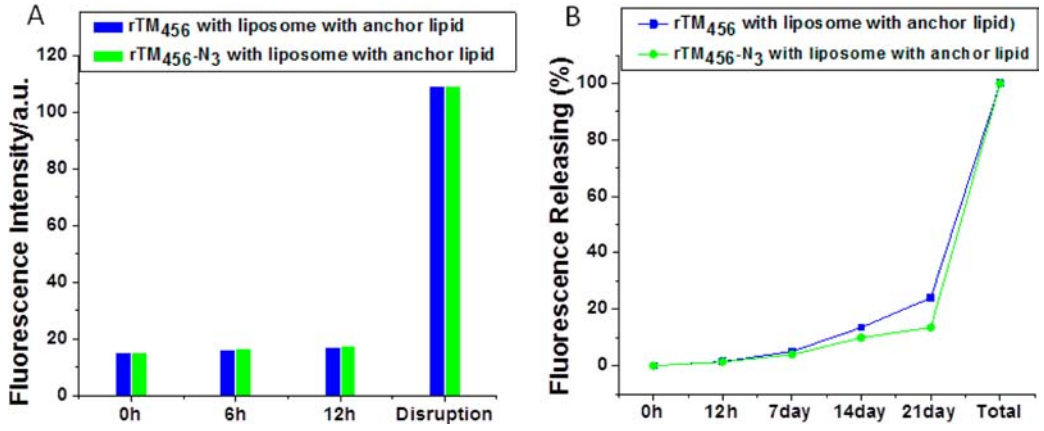

Figure 7. Evaluation of stability of liposome during copper-free click conjugation reaction (A) and stability of rTM_{456}-liposome conjugate (B) by monitoring fluorescent dye 5,6-CF releasing from liposomes. Disruption of liposomes was conducted by adding PBS buffer containing 0.5% Triton X-100 surfactant into the liposome solutions at the final point. Total is the total amount of 5,6-CF determined after disruption of liposome.

Western blotting confirmed the new band formed upon DsbA-His_6-Stag-rTM_{456}-N_3 reacted with the liposome bearing the anchor lipid DBCO-PEG_{2000}-DSPE (Figure 5A). As in the same procedure, the targeted rTM_{456}-liposome conjugate was synthesized by reacting the rTM_{456}-N_3 with liposomes bearing the anchor lipid DBCO-PEG_{2000}-DSPE in Tris-HCl buffer. As shown in Figure 5B, Western blotting confirmed the new band formed upon the rTM_{456}-N_3 reacted with the liposome. Both reactions were monitored for 9 h, which indicated that no more products formed after that time. On the other hand, the control experiment between rTM_{456} without azide and liposome with anchor lipid did not exhibit any new band formed (Figure 3C). These results indicated successful liposomal modification of the rTM_{456}-N_3 via copper-free click chemistry.

The conjugation efficiency of the copper-free click conjugation was evaluated by a given amount of rTM_{456}-N_3 and fixed anchor lipid (DBCO-PEG_{2000}-DSPE) concentration in the liposome (2% of the lipids in liposome). The grafted amount of rTM_{456}-N_3 onto the liposome was determined after gel filtration purification (CL 6B, GE Healthcare) by measuring rTM_{456}-PEG_{2000}-DSPE formed and unreacted rTM_{456}-N_3 in the buffer, respectively, in which DCBO-PEG_{2000}-DSPE was in excess to rTM_{456}-N_3. Acetone protein 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 rTM_{456}-liposome conjugate formed and ~68% by measuring the unreacted rTM_{456}-N_3, respectively. Apparently, copper-free click chemistry afforded higher yield than Staudinger ligation above. DLS was used to monitor the liposomal conjugation of the rTM_{456}-N_3 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 rTM_{456}-liposome conjugate from the reaction solution, the rTM_{456}-liposome conjugate did not show size change (Figure 6C). Furthermore,

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Table 1. Protein C Activation Activity of rTM_{456}-N_3 and Its Conjugates

	full TM[a]	rTM_{456}-N_3[b]	Tags-rTM_{456}-N_3	rTM_{456}-N_3	rTM_{456}–Liposome (SL)	rTM_{456}–Liposome (CFCC)
K_m (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
k_{cat} (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
k_{cat}/K_m ($min^{-1}.\mu M^{-1}$)	0.37 ± 0.14	0.16 ± 0.05	0.40 ± 0.15	0.29 ± 0.11	0.77 ± 0.26	0.71 ± 0.32

[a]Commercial human full TM. [b]Protein C activation activity of rTM_{456}-N_3 reported by Cazalis et al.,[20] Tags-rTM_{456}-N_3: DsbA-His_6-Stag-rTM_{456}-N_3, SL: Staudinger ligation, CFCC: copper-free click chemistry.

there was no size change of control experiment (119 ± 10 nm) in the presence of rTM_{456} 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 rTM_{456}-liposome conjugate (Figure 7A), no apparent leakage was triggered by the conjugation reaction compared with the control liposomes incubated in the presence of rTM_{456} 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 rTM_{456}-N_3 to the liposome surface may have enhanced the liposome stability as it exhibited significantly slower dye release between 14 and 21 days as compared to the control liposome treated with rTM_{456} without azide group (Figure 7B). These results indicated the compatibility of the copper-free click chemistry for rTM_{456}-liposome formation, and the rTM_{456}-liposome conjugate was quite stable and could be used for further activity evaluation.

Protein C Activation Activity of rTM_{456}-N_3 and Its Liposomal Conjugates. In this study, protein C activation activities of rTM_{456}-N_3 and its liposomal conjugates were evaluated. The activity of rTM_{456} was defined as moles of produced activated protein C per min by given amounts of rTM_{456}-N_3 and rTM_{456} conjugates in the presence of thrombin and calcium. All protein C activation assays by rTM_{456}-N_3 and rTM_{456}-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 Ca^{2+} at 37 °C, as previously reported.[40] As shown in Table 1, rTM_{456}-liposome conjugate made via either Staudinger ligation or copper-free click chemistry showed a 2-fold higher k_{cat}/K_m value than that of either DsbA-His_6-Stag-TM_{456}-N_3 or rTM_{456}-N_3, even though there was no apparent change of k_{cat}. This change in k_{cat}/K_m mainly was due to a decreased K_m value, which represents the affinity of rTM_{456} for protein C. This result was consistent with a previous report of increased k_{cat}/K_m values due to a higher affinity of rTM_{456} for protein C. It has been know 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 bioinspired liposomal TM conjugates that were synthesized by site-specific conjugation of recombinant TM_{456}-N_3 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 and 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 rTM_{456}-liposome conjugate showed a 2-fold higher k_{cat}/K_m value for protein C activation than that of rTM_{456} alone, which indicated that the lipid membrane has a beneficiary effect on the rTM_{456}'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 TM_{456} 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.

ASSOCIATED CONTENT

Supporting Information

Data on liposome surface glyco-functionalization via copper-free click chemistry. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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