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Recombinant and Chemo-/Bio-Orthogonal Synthesis of Liposomal Thrombomodulin and Its Antithrombotic Activity

Lin Wang^{1,3}, Rui Jiang^{1,2}, Yang Liu^{1,3}, Maosheng Cheng³, Qingyu Wu⁴, and Xue-Long Sun^{1,*}

¹Department of Chemistry, Chemical and Biomedical Engineering and Center for Gene Regulation of Health and Disease (GRHD), Cleveland State University, 2121 Euclid Ave, Cleveland, OH, 44115, USA

²College of Life and Health Sciences, Northeastern University, 11 Wenhua Rd, Heping Qu, Shenyang 110004, PR China

³Key Laboratory of Structure-Based Drugs Design & Discovery of Ministry of Education, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, 103 Wenhua Rd, Shenhe Qu, Shenyang 110016, P. R. China

⁴Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Ave Cleveland, OH 44195, USA

Abstract

Thrombomodulin (TM) is an endothelial cell membrane protein that acts as a major cofactor in the protein C anticoagulant pathway. The EGF-like domains 4–6 of TM (TM₄₅₆) are essential for PC activation. In this study, we proposed a liposomal recombinant TM conjugate to mimic the membrane TM structure and its anticoagulant activity. First, a DSPE-PEG₂₀₀₀-TM₄₅₆ was successfully synthesized by site-specific conjugation of azido-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO *via* copper-free click chemistry quantitatively. Then, liposome-TM₄₅₆ was fabricated *via* direct liposome formation with the DSPE-PEG₂₀₀₀-TM₄₅₆ and other lipids. This liposomal formulation of TM₄₅₆ retained protein C activation activity as that of TM₄₅₆. Also, liposome-TM₄₅₆ was much more stable and had a longer plasma half-life than TM₄₅₆ and DSPE-PEG₂₀₀₀-TM₄₅₆, respectively. Moreover, liposome-TM₄₅₆ showed *in vivo* anticoagulant effect by decreasing the mortality from 80% to 20% in a thrombin-induced thromboembolism mouse model. The reported liposome-TM₄₅₆ conjugate mimics the endothelial TM anticoagulation activity and may serve as an effective anticoagulant agent candidate for future development.

Keywords

thrombomodulin; protein C; thrombin; thromboembolism; liposome; click chemistry

*Corresponding author. Xue-Long Sun, Tel: +1-216-687-3919; Fax: +1-216-687-9298; x.sun55@csuohio.edu (X.-L. Sun).

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Thrombomodulin (TM) is an endothelial anticoagulant protein that plays important roles in controlling hemostatic balance (1). TM modulates the activity of thrombin from a procoagulant to an anticoagulant protease. When bound to TM, thrombin is unable to cleave fibrinogen or activate platelets. Instead, it activates protein C (PC) to activated PC, which is an anticoagulant protease and selectively inactivates coagulation factors Va and VIIIa, providing an essential feedback mechanism in preventing excessive coagulation (1). Recent studies confirmed that TM also has antifibrinolytic activity by activating thrombin activatable fibrinolysis inhibitor (TAFI) (2), also known as carboxypeptidase B2 (3). It has been found that the EGF-like domains 3–6 of TM are required for TAFI activation, whereas the EGF-like domains 4–6 of TM (TM₄₅₆) are essential for PC activation (4). Therefore, recombinant TM₄₅₆ may serve as a pure activated protein C (APC) generating agent for anticoagulant applications.

Reconstitution of membrane proteins into liposome is a useful technique to study the functions of the membrane proteins (5). Incorporate TM into liposome has been attempted for studying TM functions and lipid effect (6). Horie, *et al.* demonstrated that native TM incorporated into lipid vesicles resulted in substantially enhanced PC activation (7). Recently, Chaikof, *et al.*, reported that full rabbit TM directly constituted into liposome enhanced early syngeneic islet engraftment by reducing the instant blood-mediated inflammatory reaction (IBMIR) (8), and improved early outcomes after intraportal islet transplantation (8,9). More recently, we reported a liposomal TM conjugate by using post-conjugation approach of liposome preparation, and this TM conjugate showed promising *in vitro* antithrombotic activity (10). However, the TM conjugation yields were low by this method. In this study, we proposed a liposome-TM₄₅₆ conjugate *via* a direct liposome formation with distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-PEG₂₀₀₀-TM₄₅₆ (Figure 1). First, the DSPE-PEG₂₀₀₀-TM₄₅₆ was synthesized by site-specific conjugation of azido-TM₄₅₆ with DSPE-PEG₂₀₀₀-dibenzocyclooctyne (DBCO) *via* copper-free click chemistry. Then, liposome-TM₄₅₆ was fabricated with the DSPE-PEG₂₀₀₀-TM₄₅₆ and dipalmitoylphosphatidylcholine (DPPC) and cholesterol. In order to prepare the long-circulation liposome, PEG₂₀₀₀ linker was utilized, and the size of the liposome was monitored to ensure that it matched the size range of long-circulation liposome. We investigated *in vitro* and *in vivo* antithrombotic activities of this liposome-TM₄₅₆ conjugate. First, the *in vitro* protein C activity of liposome-TM₄₅₆ conjugate was investigated to determine whether this new formulation affects TM₄₅₆ anticoagulant activity. Further, *in vitro* plasma stability and *in vivo* antithrombotic effect of the liposome-TM₄₅₆ conjugate were investigated with a thrombin-induced thromboembolism mouse model. Finally, thrombin clotting time was measured over time to the therapeutic efficacy of the liposomal formulation of TM₄₅₆. The reported liposome-TM₄₅₆ mimics the native endothelial TM antithrombotic mechanism associated with cell membrane lipid components. The biomimetic liposome-TM₄₅₆ conjugate is expected to be a more efficacious agent than current soluble TM without the membrane domain, and thus may serve as an effective anticoagulant candidate for future anticoagulant development.

Materials and Methods

Materials

C57BL/6 mice (male 6–8 weeks old) were from Charles River Laboratories International, Inc.. Mouse and human plasma samples were from Innovative Research (Novi, MI). Human protein C, human thrombin and human antithrombin III were from Haematologic Technologies Inc.. Chromogenic thrombin substrate BIOPHEN-CS01 was from Aniaara. DPPC and DSPE-PEG₂₀₀₀-DBCO were from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was 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 from ImmunoChemistry Technologies, LLC (Bloomington, MN). The mouse anti-TM monoclonal antibody PBS-01 was from Abcam (Cambridge, MA). THETM DYKDDDDK Tag antibody [Biotin] (mAb, Mouse) and Streptavidin-HRP were from GenScript, USA, Inc (Piscataway, NJ). Anti-PEG monoclonal antibody E11 was from Academia Sinica.

Expression and purification of soluble SrtA

Soluble SrtA with a C-terminal His6-tag was expressed and purified from *E. coli* BL21. Briefly, the gene encoding *S. aureus* COL SrtA- N59 (residues 60-206) was inserted into NcoI-XhoI sites of pET28b vector. The plasmid pET28b-SrtA was transformed into *E. coli* BL21 (DE3). The protein expression was induced by IPTG at a final concentration of 0.5 mM. After 4 h growth at 37°C, the cells were harvested by centrifugation and lysed by sonication. The extract was centrifuged at 20,000 g for 20 min, and the supernatant containing SrtA was purified by 5 mL HiTrap Ni column (GE Healthcare). The fractions containing rSrtA were collected and then dialyzed against 20 mM Tris and 150 mM NaCl, pH 8.0, and stored at –80°C until use.

Azido-TM₄₅₆ preparation via sortase A-mediated ligation

TM₄₅₆-azido was prepared *via* sortase A (SrtA)-mediated ligation (SML) by recombinant TM₄₅₆ containing LPETG motif with the substrate diGly-PEG₃-azide by the method developed in our previous report (11). Briefly, The gene encoding the EGF domain 4–6 of human TM (TM₄₅₆) with a FLAG tag and a C-terminal LPETG motif was designed and synthesized (Genscript Inc.). The TM₄₅₆ gene was then transferred into pET-28b vector and transformed into *E. coli* B834 (DE3) cells for expression. The TM₄₅₆ was purified by HisTrap FF column (GE Healthcare). The TM₄₅₆ containing LPETG motif (10 μM) was mixed with SrtA (5 μM) and diGly-PEG₃-azide (100 μM) in reaction buffer (20 mM Tris, 150 mM NaCl and 5 mM CaCl₂, pH 8.0). The reaction mixture was incubated at 37°C for 2 h to generate the C-terminal modified TM₄₅₆. Excess diGly-PEG₃-azide was removed by a HiTrap desalting column (GE Healthcare). The obtained azido-TM₄₅₆ was further purified from the remaining mixture by collecting the pass through fraction of HisTrap FF column.

Synthesis of DSPE-PEG₂₀₀₀-TM₄₅₆ and western blot analysis

Azido-TM₄₅₆ and DSPE-PEG₂₀₀₀-DBCO were mixed in a reaction solution (20 mM Tris and 150 mM NaCl, pH 7.8) at final concentrations of 10 μM and 200 μM, respectively. The

reaction solution was incubated at room temperature overnight. The excess unreacted DSPE-PEG₂₀₀₀-DBCO was removed by ANTI-FLAG[®] M1 Agarose Affinity Gel (Sigma-Aldrich) which binds with TM₄₅₆ containing FLAG tag. The successful formation of DSPE-PEG₂₀₀₀-TM₄₅₆ was confirmed in a 15% SDS-polyacrylamide gel under reducing conditions and transferred onto a polyvinylidene difluoride membrane by Trans-Blot[®] Turbo[™] Transfer system (Bio-Rad, Hercules, CA). The membrane was blocked in 5% non-fat milk in TBS/Tween 20 (0.05%) at room temperature for 1 h with gentle shaking. Primary anti-TM monoclonal antibody (1:500) or anti-PEG monoclonal antibody E11 (1:1000) in TBS/Tween 20 (0.05%) and 2% non-fat milk (10 mL) was incubated with the membrane at 4 °C overnight. The membrane was then washed with TBS/Tween 20 (0.05%) (4×15 minutes) and then incubated with a secondary antibody (goat anti-mouse HRP 1:2000) in TBS/Tween 20 (0.05%) for 1 h at room temperature. After washing with TBS/Tween 20 (0.05%) (4×15 minutes), protein bands on the membrane were visualized using an enhanced chemiluminescence (ECL) solution (GE Healthcare Bio-Sciences, Pittsburgh, PA).

Preparation of liposome-TM₄₅₆ conjugate

DPPC (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 in liquid N₂ and a 65 °C water bath were performed. DSPE-PEG₂₀₀₀-TM₄₅₆ (30 nmol) was added to the PBS solution containing DPPC and cholesterol, and incubated for 1 h 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. Extra DSPE-PEG₂₀₀₀-TM₄₅₆ was removed by gel filtration (CL 6B, GE Healthcare). The plain liposome was prepared with a similar method by replacing the DSPE-PEG₂₀₀₀-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO.

The size of plain liposome and liposome-TM₄₅₆ was determined by DLS (dynamic light scattering), using 90plus particle size analyzer (Brookhaven Ins. Co., USA). All measurements were performed at 25 °C with a measuring angle of 90° to the incident beam. The values presented were the mean and standard deviation of three replicate samples (Table S1).

Quantitation of DSPE-PEG₂₀₀₀-TM₄₅₆ in liposome formulation

To quantify DSPE-PEG₂₀₀₀-TM₄₅₆ in liposome, sandwich ELISA was explored by utilizing an anti-thrombomodulin antibody as a capture antibody and an anti-FLAG-Tag antibody as a detection antibody. First, anti-thrombomodulin mAb (5 μg/mL) in 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 blocker for 10 h at room temperature. TM₄₅₆ standards [0–40 ng/mL] were prepared in Neptune sample diluent. In order to measure the total amount of DSPE-PEG₂₀₀₀-TM₄₅₆ in liposome, liposome was broken by detergent Triton X-100 before measurement. Standards and samples were incubated overnight at 4 °C and 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. Finally, OD at 450 nm was measured, and concentrations of DSPE-PEG₂₀₀₀-TM₄₅₆ in liposome were then extrapolated from the linear equation based on the corresponding standard curve.

Evaluation of the stability liposome-TM₄₅₆

The stability of liposome-TM₄₅₆ conjugate and plain liposome were monitored by measuring fluorescent leakage by using encapsulated self-quenching 5,6-carboxyfluorescein. Briefly, the lipids film containing the same component as mentioned in section “Preparation of liposome-TM₄₅₆ conjugate” was swelled in the dark with 2 mL PBS containing 85 mM 5,6-Carboxyfluorescein (5,6-CF). Ten freeze-thaw cycles in liquid N₂ and a 65 °C water bath were performed. DSPE-PEG₂₀₀₀-TM₄₅₆ (30 nmol) was added to the PBS solution containing DPPC and cholesterol, and incubated for 1 h in room temperature with shaking in the dark. Then the mixture was extruded through polycarbonate membranes (Millipore size from 400 nm and 100 nm) to produce small unilamellar vesicles. Separation of the CF vesicles from non-entrapped CF was achieved by gel filtration chromatography (Sephadex G-50). The plain liposome was prepared with a similar method by replacing the DSPE-PEG₂₀₀₀-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO. The fluorescence intensities were monitored for four days. On the last day, 0.5 % Triton X-100 PBS solution was added into sample above to release all entrapped CF molecules from the liposomes.

Protein C activation activity assay

The cofactor activities of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates were assessed by a protein C activation assay. Azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates, as described 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 activation was 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 absorbance at 405 nm were measured after 20 min incubation.

Stability of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates in mouse plasma

Azido-TM₄₅₆ or liposome-TM₄₅₆ 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-TM₄₅₆ and liposome-TM₄₅₆ conjugates were measured by the sandwich ELISA. TM₄₅₆ standards in plasma were prepared by spiking with mouse plasma at a concentration of 8 µg/mL, which were diluted by Neptune sample diluent to target concentrations [0–40 ng/mL]. Azido-TM₄₅₆ and TM₄₅₆ conjugate samples in mouse plasma for stability study were prepared similarly to TM₄₅₆ standards in plasma. Then the same sandwich ELISA protocol was followed as in section “Quantitation of DSPE-

PEG₂₀₀₀-TM₄₅₆ in liposome formulation” to measure the concentrations of azido-TM₄₅₆ and TM₄₅₆ conjugates in plasma based on the corresponding standard curve.

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

Animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health (NIH; Bethesda, MD, USA), under protocols 21113-SUN-AS, approved by the Cleveland State University Institutional Animal Care and Use Committee. Male C57BL/6 mice of 42–56 days of age (Charles River, Wilmington, MA, USA) were used for all experiments.

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 liposome-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 surviving mice were euthanized.

Thrombin clotting time (TCT)

C57BL/6 mice were dosed with 3 mg/kg azido-TM₄₅₆ and liposome-TM₄₅₆ 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 sodium citrate medium (9 parts blood, 1 part anticoagulant). The collected blood was then centrifuged at 1500 × g for 10 min to obtain the plasma. The plasma (100 µL) 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-TM₄₅₆ or liposome-TM₄₅₆ derivatives.

For the concentration dependent TCT assay, 25 µL of azido-TM₄₅₆ or liposome-TM₄₅₆ 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.

Blood concentration measurement of TM₄₅₆ in mice

Azido-TM₄₅₆ (3 mg/kg) or equivalent molar amount of liposome-TM₄₅₆ was injected to C57BL/6 mice through the tail vein, and the blood was collected at different time points. For each time point, three mice were sacrificed. Concentrations of azide-TM₄₅₆ and liposome-TM₄₅₆ in blood were then measured by ELISA as mentioned above.

Calculation of protein concentrations

Concentrations and doses were calculated based on protein content using the same molecular weight (16 kDa) for TM₄₅₆, DSPE-TM₄₅₆ and liposome-TM₄₅₆. Hence, at a given mg/mL concentration or mg/kg dose, equimolar amounts of the molecules were used.

Results and Discussion

Synthesis of liposome-TM₄₅₆ conjugate via direct liposomal formation

Liposome-protein conjugate (proteoliposome) serves as a practical biomimetic system to study membrane protein structure and function (12). In general, three methods have been used to prepare liposome-protein conjugate. 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 (13,14). However, it requires a relatively high temperature, which may reduce protein 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 and lower coupling yields (15). Moreover, the liposome size may alter after the conjugation, which affects the clearance behavior *in vivo* (16). In our previous study (10), TM₄₅₆ was incorporated to the liposome by post-functionalization method, in which DBCO-PEG₂₀₀₀-DSPE was used as the reactive functionalities on the liposome to conjugate with azido-TM₄₅₆ through copper-free click chemistry. Due to the poor solubility of DBCO group, the percentage of available anchor lipid in liposome is limited that affected the efficiency of liposome-TM₄₅₆ conjugation. In this study, in order to maximally maintain the activity of TM₄₅₆ and avoid the limitation of poor solubility of DBCO group, we investigated direct liposome formation method, in which the lipidated protein (DSPE-PEG₂₀₀₀-TM₄₅₆) is synthesized first and then mixed with other lipids directly to form the liposome-TM₄₅₆ conjugate.

First, a recombinant TM₄₅₆ with one azide functionality at the C-terminus (azido-TM₄₅₆) was prepared *via* sortase A-mediated ligation (SML) by expressing the LPETG motif containing TM₄₅₆, with the catalysis of sortase A developed in our previous report (11). The substrate GlyGly-PEG₃-azide was conjugated to the C-terminal of TM₄₅₆ developed in our previous report (11). This is a site-specific modification of TM₄₅₆, resulting in only one azide group on the C-terminal. Moreover, it revealed that SML offers the highest overall yield of TM₄₅₆-azido comparing with other chemical modification methods or unnatural amino acid expression (incorporation of azide group to protein during expression) (11). Therefore, SML-mediated incorporation of azide facilitates large amount of azido-TM₄₅₆ generation for the liposomal-TM₄₅₆ preparation, especially for the *in vivo* study.

Next, DSPE-PEG₂₀₀₀-TM₄₅₆ was prepared by site-specific conjugation of azido-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO *via* copper-free click chemistry (CFCC) (Figure 2A). The PEG₂₀₀₀ chain between the lipid and protein was used in our liposome-TM system as it may serve as to stabilize both the protein and liposomes. Protein PEGylation molecule is commonly used to enhance the pharmacokinetic properties of protein drug due to the effects of reducing glomerular filtration rate, mononuclear phagocyte system (MPS) uptake and the immune response (17). Besides directly linking with drug, the PEG molecule was also investigated to improve other delivery systems, such as covering the liposome with PEG molecule to form long-circulating liposomes or stealth liposome (18). The new DSPE-PEG₂₀₀₀-TM₄₅₆ was

confirmed by SDS-PAGE and western blot analysis. A new band showed up around 30 kDa (Figure 2B Lane 2) and the change of molecular weight was as expected since the molecular weight of DSPE-PEG₂₀₀₀-DBCO is around 3 kDa. This indicated the successful conjugate formation. Further, the original band for pure azido-TM₄₅₆ disappeared completely, indicating a quantitative conjugation, in which all the azido-TM₄₅₆ reacted with DSPE-PEG₂₀₀₀-DBCO. To further confirm this new band is the DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate, western blotting was performed by using an anti-PEG antibody which recognizes PEG chain longer than 2000 units and an anti-TM antibody that binds with TM₄₅₆ (Figure 2B). The newly formed band was observed for both antibodies (Figure 2B, Lane 2), indicating the new DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate formation.

With the DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate in hand, liposome-TM₄₅₆ conjugate was then prepared *via* direct liposome formation 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 film. After re-suspended in PBS buffer and freeze-thaw processes, the DSPE-PEG₂₀₀₀-TM₄₅₆ was added to the PBS solution containing DPPC and cholesterol, and incubated for one hour at room temperature with shaking. Then, the mixture was extruded through 400 nm and 100 nm polycarbonate filters, respectively. The plain liposome was prepared by the same process except by using DSPE-PEG₂₀₀₀-DBCO instead of DSPE-PEG₂₀₀₀-TM₄₅₆. Gel filtration was conducted to remove the un-incorporated DSPE-PEG₂₀₀₀-TM₄₅₆, then, the amount of total incorporated TM₄₅₆ in liposome was measuring by ELISA. The incorporating yield was then calculated as ~86%. Compared with the previous method using post-modification through copper-free click chemistry whose yield is ~60% (10), the present method enhanced the incorporating yield by more than 20%. A main reason for the low incorporating yield of previous post-conjugation method was the hydrophobicity of DBCO group which is likely buried between the lipid bilayer of the liposome, leading to little amount of DBCO group available for reacting with azide containing TM₄₅₆.

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 concordance with the extrusion by 100 nm filter and within the size range for long circulation *in vivo*. The stability of liposome-TM₄₅₆ was also determined for four days by measuring fluorescent leakage (Figure 3). Compared with plain liposome, there was no apparent leakage for liposome-TM₄₅₆ during four days period, indicating that the anchoring of DSPE-PEG₂₀₀₀-TM₄₅₆ didn't affect the liposome stability.

Protein C activation activity of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugate

In order to know whether the liposome modification to TM₄₅₆ affects TM₄₅₆'s anticoagulant activity, protein C activation activities of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates were measured. The absorptions of activated protein C (APC) cleaved chromogenic substrates were measured at 405 nm. The rate of activating PC by thrombin was slow in the absence of TM₄₅₆ (Figure 4, column 1), whereas in the presence of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugate, the generation of APC was significantly enhanced (Figure 4, column 2–5). As shown in Figure 4 after the conjugation of DSPE-PEG₂₀₀₀-DBCO and TM₄₅₆.azido, there

was no significant change in PC activation activity, indicating that the lipidation didn't hamper the TM₄₅₆ activity. These results demonstrated that the site specific lipidation of TM₄₅₆ did not affect its anticoagulant activity, even using a two-step reaction involving SML and CFCC. In contrast, the activity of liposome-TM₄₅₆ was ~30% lower than that of both azido-TM₄₅₆ and DSPE-PEG₂₀₀₀-TM₄₅₆. This may be caused by the liposomal formulation of DSPE-PEG₂₀₀₀-TM₄₅₆ that entraps part of TM₄₅₆ facing inside the liposome. Therefore, this part of TM₄₅₆ was blocked by the bilayer of liposome to interact with thrombin and protein C in the solution. To test this hypothesis, Triton X-100 was used as detergent to break the liposome. As a result, all of the DSPE-PEG₂₀₀₀-TM₄₅₆ molecules 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-TM₄₅₆ increased to the similar level as azido-TM₄₅₆. In order to exclude the effect of Triton X-100, the activities of TM₄₅₆-N₃ and DSPE-PEG₂₀₀₀-TM₄₅₆ w/and w/o Triton X-100 were measured. It showed that there was no significant difference between the groups in the presence of Triton X-100 or not (Table S2). All of these suggested that the liposomal formulation did not affect the activity of TM₄₅₆, and the difference in activities between the assays with and without Triton X-100 should correspond to the TM₄₅₆ encapsulated inside the liposome.

Plasma stability of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates

Plasma stability has significant implications in drug development, especially for protein-based drugs. Drugs with poor plasma stability often show short half-lives, which further impact the distribution and efficacy of the drug. Blood contains many proteolytic enzymes, which hydrolyze protein and peptides, thereby reducing protein stability in plasma (19–22). For this reason, the *in vitro* plasma stability of azido-TM₄₅₆ and TM₄₅₆ conjugates were investigated. Briefly, azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates were incubated with mouse plasma at 37 °C for different time periods, and the changes of protein concentration were measured by sandwich ELISA. As shown in Figure 5, azido-TM₄₅₆ degraded to less than 40% after 3 h, whereas DSPE-PEG₂₀₀₀-TM₄₅₆ and liposome-TM₄₅₆ conjugates showed much slower degradation. Even after 24 h, there was still more than 50% of the DSPE-PEG₂₀₀₀-TM₄₅₆ remaining in the plasma. This was likely due to the protective effect of the PEG₂₀₀₀ molecule between the lipid and protein. PEGylation is a well-known approach to improve drug's pharmacokinetic properties, mainly owing to a reduction of clearance rate by the kidney and proteolysis (23,24). As for this *in vitro* assay, the prominent effect of the PEG₂₀₀₀ chain as well as the lipid should be the steric hindrance (25) that blocks the hydrolysis of TM₄₅₆ by plasma proteases. Specifically, because of the exopeptidases in plasma, modification of *N*- or *C*-terminal of proteins provides an efficient way to increase enzymatic stability (26). It was also observed that liposome-TM₄₅₆ conjugate had a longer *in vitro* clearance time in plasma than DSPE-PEG₂₀₀₀-TM₄₅₆. Besides the reason of PEG chain protection mentioned above, this enhancement of *in vitro* stability in plasma may be due to the partial TM₄₅₆ facing inside the liposome, which cannot be accessed by the proteases, thereby leading to better stability than DSPE-PEG₂₀₀₀-TM₄₅₆.

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

The *in vivo* antithrombotic effect of liposome-TM₄₅₆ 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 (27,28). After human thrombin injection, the mice die within 15 minutes due to acute fibrin deposition within the pulmonary microvascular system (27,28). 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-TM₄₅₆ and liposome-TM₄₅₆ conjugates were injected into the mouse tail vein three minutes prior the injection of human thrombin in order to protect mice from thromboembolism. The control group injected with PBS buffer only before human thrombin had a similar mortality rate ~80% (Figure 6). For the azido-TM₄₅₆ group, three different doses (0.2, 0.4, and 0.8 mg/kg) were investigated. At the dose of 0.2 mg/kg, there was no apparent anti-thrombotic effect for the mice, since the mortality was still ~80%. When the dose was increased, the mortality decreased from ~80% to ~20% (Figure 6). It was noticed that 0.8 mg/kg 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 prevented all the mice from the acute thromboembolism (27). This suggested that synthesized azido-TM₄₅₆ can achieve an anti-thrombotic effect comparable to that of TMD123.

Next, the anti-thrombotic effect of liposome-TM₄₅₆ conjugate was determined together with the plain liposome as a control. The plain liposome did not have any effects in this thromboembolism mouse model with a mortality of ~80% (Figure 6). The same doses of azido-TM₄₅₆ were used for liposome-TM₄₅₆, and it showed a similar trend as azido-TM₄₅₆. 0.2 mg/kg caused ~80% mortality, 0.4 mg/kg caused ~40% mortality and 0.8 mg/kg caused ~20% mortality, indicating that the liposome-TM₄₅₆ conjugate achieved the same anticoagulant effect as azido-TM₄₅₆ (Figure 6). Even though the *in vitro* anticoagulant assay suggested that there was around 30% reducing in activity of unbroken liposome-TM₄₅₆ than azido-TM₄₅₆ due to the partial TM₄₅₆ inside the liposome, the *in vivo* anticoagulant effect of liposome-TM₄₅₆ remained the same as azido-TM₄₅₆. This is probably because of the differences *in vivo* behaviors between free and liposome conjugated proteins (29).

Prolongation of thrombin clotting time by liposome-TM₄₅₆ conjugate

In order to determine the effect of liposome-TM₄₅₆ conjugate on thrombin clotting time (TCT), both the *in vitro* and *in vivo* TCT was measured for pure azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates. Different concentrations of azido-TM₄₅₆ or liposome-TM₄₅₆ conjugate were mixed with plasma, and then human thrombin was added to trigger the clotting. For both azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates, increasing the concentration prolonged the clotting time (Figure 7A), showing a direct positive 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-TM₄₅₆ and liposome-TM₄₅₆ conjugates. But at higher concentrations, the

difference between the two became obvious. For azido-TM₄₅₆, a concentration of 0.05 mg/mL doubled the clotting time, whereas twice of that concentration, 0.1 mg/mL of liposome-TM₄₅₆ conjugate reached a similar antithrombotic effect (Figure 7A). The lower antithrombotic activity of liposome-TM₄₅₆ conjugate compared with azido-TM₄₅₆ was consistent with the APC activities, which was also due to the fact that a part of TM₄₅₆ was encapsulated in the liposome or facing the inside of the liposome, lacking contacts with thrombin and protein C in the plasma. In addition, it is possible that the lipid part of liposome-TM₄₅₆ interacted with one or some of the factors involved in the clotting pathway in the plasma, resulting in the change of activity of liposome-TM₄₅₆ in two assays. More research will be conducted to find out this kind of interaction in the future.

Next, the *in vivo* effect of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates on TCT in mouse at different time points was investigated. Three mg/kg of azido-TM₄₅₆ or equivalent molar amount of liposome-TM₄₅₆ conjugate was injected into C57Bl/6 mice *via* tail vein, and blood was collected at 0, 0.5, 1, 3, and 6 h. The blood contained no azido-TM₄₅₆ or liposome-TM₄₅₆ conjugate was also collected as a control for TCT. TCT was then measured by mixing the human thrombin with the citrated mouse plasma. The TCT value was 24.8 ± 1.0 s in controls. The prolongation of TCT was calculated by subtracting the control clotting time from TCT in experiment groups. As shown in Figure 7B, the prolongation of TCT decreased from more than 40 sec to less than 5 sec in a time-dependent manner, indicating the decrease of concentrations of azido-TM₄₅₆ or liposome-TM₄₅₆ conjugate in the plasma. For azido-TM₄₅₆, the prolongation of TCT decreased to less than 10 s at 30 min, which was less than 15% of the initial prolongation of TCT, suggesting that the concentration of azido-TM₄₅₆ dropped by a similar rate. Consistent with our results, TM₄₅₆ was reported to have half-lives of around 6–9 min in rats and monkeys (30).

Liposome-TM₄₅₆ conjugate demonstrated a quite different blood profile, showing a slower reduction in TCT prolongation compared with azido-TM₄₅₆. At 30 min, the prolongation of TCT was still around 38 sec, which was almost five times longer than that with azido-TM₄₅₆. It indicated that there was more TM₄₅₆ in liposomal formulation than free protein at 30 min, since there was a direct relationship between TM concentrations and TCT. Till 6 h, the prolongation of TCT dropped to around 8 sec which was the similar level of azido-TM₄₅₆ at 30 min. In order to confirm the TCT change was caused by the varying concentration of TM₄₅₆ in blood, the concentrations of azido-TM₄₅₆ and liposome-TM₄₅₆ in blood were measured by ELISA, and it showed a similar trend for each form of TM₄₅₆ as the TCT test (Fig. S1). It suggested that liposomal formulation enhanced the circulation time and prolonged the time of anticoagulant effect of TM₄₅₆.

TM₄₅₆ is different from human soluble TM that has anticoagulant activity as well as the coagulant activity *via* the thrombin activatable fibrinolysis inhibitor (TAFI) activation by domain EGF3–6. Therefore TM₄₅₆ has little procoagulant activity and will be a potential candidate as pure anticoagulant. However, the main drawback of TM₄₅₆ is the short half-life, only 6–9 min in rats and monkeys (31), and this disadvantage limits the application of TM₄₅₆ as a protein drug. A half-life of only few minutes is not sufficient for drug to reach the target tissues or organs with effective levels. This is commonly caused by the fast renal clearance related to the hydrophilic nature and the small size of the protein drug, or the

enzymatic degradation by enzymes in blood (25). After incorporating the TM₄₅₆ into liposome, it showed an increase in half-life based on the effect of prolongation of TCT, still with the same *in vitro* protein C activation activity (not clear). Several factors may contribute to this improvement in the half-life. First of all, the conjugation of long PEG chain and lipid at the C-terminal of TM₄₅₆ protected the degradation by enzymes in blood. Secondly, after the incorporation of DSPE-PEG₂₀₀₀-TM₄₅₆ into liposome, a part of TM₄₅₆ was buried inside the liposome, which also offers the protection for this part of TM₄₅₆ by the lipid bilayer. Most importantly, the liposomal formulation altered the clearance pathway and resulted in a prolonged anticoagulant effect in mice.

In summary, a lipidated TM₄₅₆, DSPE-PEG₂₀₀₀-TM₄₅₆ was successfully synthesized by site-specific conjugation of recombinant azido-TM₄₅₆ with DSPE-PEG₂₀₀₀-DBCO *via* copper-free click chemistry. Then, the liposome-TM₄₅₆ conjugate was prepared by using the DSPE-PEG₂₀₀₀-TM₄₅₆ *via* direct liposome formation together with other lipids, and the size of the obtained liposome was around 130 nm. This liposomal formulation of TM₄₅₆ retained a similar protein C activation activity to that of the unmodified TM₄₅₆, and was much more stable in plasma than azido-TM₄₅₆ and DSPE-PEG₂₀₀₀-TM₄₅₆. Liposome-TM₄₅₆ conjugate also showed a comparable anticoagulant effect in thrombin-induced thromboembolism mouse model with 60% reduction in mortality. Moreover, liposome-TM₄₅₆ conjugate enhanced the circulation time, as indicated by a longer duration of the prolonged TCT as compared to that with free azido-TM₄₅₆. Overall, the liposome-TM₄₅₆ conjugate may serve as an effective anticoagulant agent candidate for future development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Liposomal thrombomodulin conjugate was fabricated via direct liposome formation with recombinant TM-lipid.
- Liposomal thrombomodulin conjugate showed potent anticoagulant effect in thrombin-induced thromboembolism mouse model.
- Liposomal thrombomodulin conjugate showed increased circulation time compared to that of recombinant thrombomodulin.

FIG. 1. Membrane mimetic synthesis for liposome-TM₄₅₆ conjugates *via* recombinant, copper-free click chemistry and direct liposome formulation.

FIG. 2.

Synthesis of DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate *via* copper-free click chemistry. **(A)** Scheme of DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate synthesis *via* copper-free click chemistry. **(B)** SDS-PAGE and western blot analysis of the formation of DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate (Lane 1: Azido-TM₄₅₆; Lane 2: DSPE-PEG₂₀₀₀-TM₄₅₆). The conjugate was detected by anti-TM antibody and anti-PEG antibody which recognizes PEG chain in DSPE-PEG₂₀₀₀-TM₄₅₆ conjugate.

FIG. 3. Stability of liposome-TM₄₅₆ conjugate 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 after disruption of liposome.

FIG. 4.

Protein C activation activity of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates. Triton X-100 was used to break the liposome and release all the DSPE-PEG₂₀₀₀-TM₄₅₆. Data points represent the mean $\pm$ SD, n=3. Control group contained no TM₄₅₆ at all. ** $P < 0.01$ vs. azido-TM₄₅₆ by unpaired Student's t-tests (two-sided).

FIG. 5.

In vitro stability of azido-TM₄₅₆ and TM₄₅₆ conjugates in mouse plasma. Azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates were incubated with mouse plasma at 37 °C for 0, 1, 3, 6, 12, 24 and 48 hours, and the concentration of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates were measured by sandwich ELISA by using anti-TM antibody as capture antibody and anti-FLAG antibody as detection antibody. The remaining azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates in plasma were shown in percentage assuming the amount of TM₄₅₆ is 100% at 0 h. Data represent mean $\pm$ SD, n=3. ** P < 0.01, *** P < 0.001 vs. azido-TM₄₅₆ by unpaired Student's t-tests (two-sided).

FIG. 6.

Effect of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates on thrombin-induced thromboembolism in mice. Control group was injected with PBS buffer instead of azido-TM₄₅₆. Three different doses of azido-TM₄₅₆ and liposome-TM₄₅₆ (lipo-TM₄₅₆) were used, 0.2 mg/kg, 0.4 mg/kg, and 0.8 mg/kg. The dose was calculated only based on protein component in the liposome-TM₄₅₆. The dosage for plain liposome is 2 mg/kg. Each group contained 5 mice, n=3, and the number of dead mice within one hour was counted. ** $P < 0.01$, *** $P < 0.001$ vs. Control by unpaired Student's t-tests (two-sided).

FIG. 7.

Prolongation of thrombin clotting time (TCT). (A) Effect of azido-TM₄₅₆ and liposome-TM₄₅₆ conjugates on increasing TCT *in vitro* at different concentrations. The concentration was calculated only on protein content. (B) Prolongation of TCT of azido-TM₄₅₆ (3 mg/kg) or equivalent molar amount of liposome-TM₄₅₆ after injecting to mice at different time

points. Data points represent mean $\pm$ SD, n=4, and two mice for each time point. ** $P < 0.01$, *** $P < 0.001$ vs. azido-TM₄₅₆ by unpaired Student's t-tests (two-sided).

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