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Recombinant and chemo-/bio-orthogonal synthesis of liposomal thrombomodulin and its antithrombotic activity

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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 (TM_{456}) 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-PEG_{2000}-TM_{456} was successfully synthesized by site-specific conjugation of azido-TM_{456} with DSPE-PEG_{2000}-DBCO *via* copper-free click chemistry quantitatively. Then, liposome-TM_{456} was fabricated *via* direct liposome formation with the DSPE-PEG_{2000}-TM_{456} and other lipids. This liposomal formulation of TM_{456} retained protein C activation activity as that of TM_{456}. Also, liposome-TM_{456} was much more stable and had a longer plasma half-life than TM_{456} and DSPE-PEG_{2000}-TM_{456}, respectively. Moreover, liposome-TM_{456} showed *in vivo* anticoagulant effect by decreasing the mortality from 80% to 20% in a thrombin-induced thromboembolism mouse model. The reported liposome-TM_{456} conjugate mimics the endothelial TM anticoagulation activity and may serve as an effective anticoagulant agent candidate for future development.

[**Key words:** Thrombomodulin; Protein C; Thrombin; Thromboembolism; Liposome; Click chemistry]

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 (TM_{456}) are essential for PC activation (4). Therefore, recombinant TM_{456} 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. (7) demonstrated that native TM incorporated into lipid vesicles resulted in substantially enhanced PC activation. Recently, Cui et al. (8) reported that full rabbit TM directly constituted into liposome enhanced early syngeneic islet engraftment by reducing the instant blood-mediated inflammatory reaction (IBMIR), 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-TM_{456} conjugate *via* a direct liposome formation with distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-PEG_{2000}-TM_{456} (Fig. 1). First, the DSPE-PEG_{2000}-TM_{456} was synthesized by site-specific conjugation of azido-TM_{456} with DSPE-PEG_{2000}-dibenzocyclooctyne (DBCO) *via* copper-free click chemistry. Then, liposome-TM_{456} was fabricated with the DSPE-PEG_{2000}-TM_{456} and dipalmitoylphosphatidylcholine (DPPC) and cholesterol. In order to prepare the long-circulation liposome, PEG_{2000} 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-TM_{456} conjugate. First, the *in vitro* protein C activity of liposome-TM_{456} conjugate was investigated to determine whether this new formulation affects TM_{456} anticoagulant activity. Further, *in vitro* plasma stability and *in vivo* antithrombotic effect of the liposome-TM_{456} conjugate were investigated with a thrombin-induced thromboembolism mouse model. Finally, thrombin clotting time (TCT) was measured over time to the therapeutic efficacy of the liposomal formulation of TM_{456}. The reported liposome-TM_{456} mimics the native endothelial TM antithrombotic mechanism associated with cell membrane lipid components. The biomimetic liposome-TM_{456} conjugate is expected to be a

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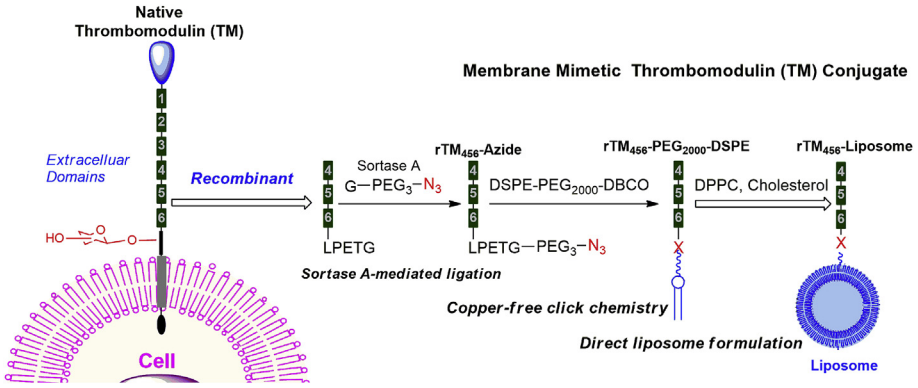

FIG. 1. Membrane mimetic synthesis for liposome-TM_{456} conjugates *via* recombinant, copper-free click chemistry and direct liposome formulation.

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. (Wilmington, MA, USA). Mouse and human plasma samples were from Innovative Research (Novi, MI, USA). Human protein C, human thrombin and human antithrombin III were from Haematologic Technologies Inc. (Essex Junction, VT, USA). Chromogenic thrombin substrate BIOPHEN-CS01 was from Aniara Diagnostica (West Chester, OH, USA). DPPC and DSPE-PEG_{2000}-DBCO were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol was from Sigma–Aldrich (St. Louis, MO, 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, USA). The mouse anti-TM monoclonal antibody PBS-01 was from Abcam (Cambridge, MA, USA). THE DYKDDDDK Tag antibody [Biotin] (mAb, Mouse) and Streptavidin-HRP were from GenScript, USA, Inc. (Piscataway, NJ, USA). Anti-PEG monoclonal antibody E11 was from Academia Sinica (Taipei, Taiwan).

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-ΔN59 (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 SrtA were collected and then dialyzed against 20 mM Tris and 150 mM NaCl, pH 8.0, and stored at −80°C until use.

Azido-TM_{456} preparation *via* sortase A-mediated ligation TM_{456}-azido was prepared *via* sortase A (SrtA)-mediated ligation (SML) by recombinant TM_{456} containing LPETG motif with the substrate diGly-PEG_3-azide by the method developed in our previous report (11). Briefly, the gene encoding the EGF domain 4–6 of human TM (TM_{456}) with a FLAG tag and a C-terminal LPETG motif was designed and synthesized (Genscript Inc.). The TM_{456} gene was then transferred into pET-28b vector and transformed into *E. coli*. B834(DE3) cells for expression. The TM_{456} was purified by HisTrap FF column (GE Healthcare). The TM_{456} containing LPETG motif (10 μM) was mixed with SrtA (5 μM) and diGly-PEG_3-azide (100 μM) in reaction buffer (20 mM Tris, 150 mM NaCl and 5 mM $CaCl_2$, pH 8.0). The reaction mixture was incubated at 37°C for 2 h to generate the C-terminal modified TM_{456}. Excess diGly-PEG_3-azide was removed by a HiTrap desalting column (GE Healthcare). The obtained azido-TM_{456} was further purified from the remaining mixture by collecting the pass through fraction of HisTrap FF column.

Synthesis of DSPE-PEG_{2000}-TM_{456} and western blot analysis Azido-TM_{456} and DSPE-PEG_{2000}-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-PEG_{2000}-DBCO was removed by ANTI-FLAG M1 Agarose Affinity Gel (Sigma–Aldrich) which binds with TM_{456} containing FLAG tag. The successful formation of DSPE-PEG_{2000}-TM_{456} 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, USA). 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 min) 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 min), protein bands on the membrane were visualized using an enhanced chemiluminescence (ECL) solution (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Preparation of liposome-TM_{456} 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 of 0.01 mM phosphate buffered saline (PBS) (pH 7.4) to form a multilamellar vesicle suspension. Ten freeze-thaw cycles in liquid N_2 and a 65°C water bath were performed. DSPE-PEG_{2000}-TM_{456} (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 of 400 nm and 100 nm) to produce small unilamellar vesicles. Extra DSPE-PEG_{2000}-TM_{456} was removed by gel filtration (CL 6B, GE Healthcare). The plain liposome was prepared with a similar method by replacing the DSPE-PEG_{2000}-TM_{456} with DSPE-PEG_{2000}-DBCO.

The size of plain liposome and liposome-TM_{456} was determined by DLS (dynamic light scattering), using 90 plus 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-PEG_{2000}-TM_{456} in liposome formulation To quantify DSPE-PEG_{2000}-TM_{456} 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. TM_{456} standards [0–40 ng/mL] were prepared in Neptune sample diluent. In order to measure the total amount of DSPE-PEG_{2000}-TM_{456} 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-PEG_{2000}-TM_{456} in liposome were then extrapolated from the linear equation based on the corresponding standard curve.

Stability of liposome-TM_{456} conjugate The stability of liposome-TM_{456} 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 the Preparation of liposome-TM_{456} conjugate section was swelled in the dark with 2 mL PBS containing 85 mM 5,6-Carboxyfluorescein (5,6-CF). Ten freeze-thaw cycles in liquid N_2 and a 65°C water bath were performed. DSPE-PEG_{2000}-TM_{456} (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 to 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-PEG_{2000}-TM_{456} with DSPE-PEG_{2000}-DBCO. The fluorescence intensities were monitored for

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