MSL ACADEMIC ENDEAVORS

Cleveland State University
EngagedScholarship@CSU

Chemistry Faculty Publications
Chemistry Department

11-1-2019

End-point Modification of Recombinant Thrombomodulin with Enhanced Stability and Anticoagulant Activity

Xia Liu
Cleveland State University

Mallorie Boron
Cleveland State University

Yu Zhao
Cleveland State University

Xue-Long Sun
Cleveland State University, x.sun55@csuohio.edu

Follow this and additional works at: https://engagedscholarship.csuohio.edu/scichem_facpub

Part of the Medicinal-Pharmaceutical Chemistry Commons

How does access to this work benefit you? Let us know!

Publisher's Statement

Link to publisher version: https://doi.org/10.1016/j.ejps.2019.105066

Recommended Citation
Liu, Xia; Boron, Mallorie; Zhao, Yu; and Sun, Xue-Long, "End-point Modification of Recombinant Thrombomodulin with Enhanced Stability and Anticoagulant Activity" (2019). *Chemistry Faculty Publications*. 525.
https://engagedscholarship.csuohio.edu/scichem_facpub/525

This Article is brought to you for free and open access by the Chemistry Department at EngagedScholarship@CSU. It has been accepted for inclusion in Chemistry Faculty Publications by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

End-point modification of recombinant thrombomodulin with enhanced stability and anticoagulant activity

Xia Liu, Mallorie Boron, Yu Zhao, Xue-Long Sun

ARTICLE INFO

Keywords:
Copper-free click chemistry
Protein C
PEGylation
Sortase A-mediated ligation
Thrombin clotting time
Thrombomodulin

ABSTRACT

Thrombomodulin (TM) is an endothelial cell membrane protein that plays essential roles in controlling vascular haemostatic balance. The 4, 5, 6 EGF-like domain of TM (TM_{456}) has cofactor activity for thrombin binding and subsequently protein C activation. Therefore, recombinant TM_{456} is a promising anticoagulant candidate but has a very short half-life. Ligation of poly (ethylene glycol) to a bioactive protein (PEGylation) is a practical choice to improve stability, extend circulating life, and reduce immunogenicity of the protein. Site-specific PEGylation is preferred as it could avoid the loss of protein activity resulting from nonspecific modification. We report herein two site-specific PEGylation strategies, enzymatic ligation and copper-free click chemistry (CFCC), for rTM_{456} modification. Recombinant TM_{456} with a C-terminal LPETG tag (rTM_{456}-LPETG) was expressed in *Escherichia coli* for its end-point modification with NH_2-diglycine-PEG_{5000}-OMe *via* Sortase A-mediated ligation (SML). Similarly, an azide functionality was easily introduced at the C-terminus of rTM_{456}-LPETG *via* SML with NH_2-diglycine-PEG_3-azide, which facilitates a site-specific PEGylation of rTM_{456} *via* CFCC. Both PEGylated rTM_{456} conjugates retained protein C activation activity as that of rTM_{456}. Also, they were more stable than rTM_{456} in Trypsin digestion assay. Further, both PEGylated rTM_{456} conjugates showed a concentration-dependent prolongation of thrombin clotting time (TCT) compared to non-modified protein, which confirms the effectiveness of these two site-specific PEGylation schemes.

1. Introduction

Thrombomodulin (TM) is an endothelial cell membrane protein that plays essential roles in controlling vascular haemostatic balance by activating anticoagulant and anti-inflammatory proteins and simultaneously inactivating pro-coagulant and pro-inflammatory proteins (Dittman and Majerus, 1990). TM serves as a receptor for thrombin and directly inhibits its pro-coagulant activity (direct anticoagulant activity). So far, various forms of TM have been cloned and recombinant expressed for structural and functional study of TM (Wang et al., 2014). Actually, various recombinant TMs show promising antithrombotic and anti-inflammatory activity *in vitro* and *in vivo* (Ma et al., 2015). Particularly, recombinant human soluble TM (ART-123) has been approved in Japan for DIC treatment (Itoh et al., 2016). However, a phase III trial of ART-123 for DIC in USA was withdrawn in 2019 (van der Poll, 2019). It is speculated that recombinant human soluble TM has many domains that have different activities. The last three consecutive EGF-like domains (E456) are responsible for the anticoagulant activity of TM (Zushi et al., 1989). Specifically, the E56 domains of TM bind thrombin, while promoting protein C binding to the E45 domains of TM, together converting protein C to activated protein C (APC) (Fuentes-Prior et al., 2000). Meanwhile, TM binding prevents the binding of thrombin to its pro-coagulant substrates, such as fibrinogen and coagulation factors V and VIII. Therefore, recombinant EGF-like domains 4, 5, 6 of TM (TM_{456}) is a promising anticoagulant candidate. However, its half-life is very short (6–9 min) in animal models (Suzuki et al., 1998).

Modification of protein can expand the protein's pharmacodynamic and pharmacokinetic properties, such as functional capacity and stability (Pelegri-O'Day et al., 2014). Covalent conjugation of polyethylene glycol (PEG) molecule to protein has been used successfully in approved protein drugs (Turecek et al., 2016). The key point for a practical protein modification is to carry out site-specific chemistry to avoid upsetting the protein's activity due to random modification (Dozier and Distefano, 2015; Nischan and Hackenberger, 2014). Bio-orthogonal

chemistry has been developed for selective and efficient modification of proteins (Willems et al., 2011). The most widely used approaches are azide-reactive Staudinger ligation (Cazalis et al., 2004) and click chemistry (Agard et al., 2006). These approaches benefit from the azide functionality since it is small, bears no overall charge and can be introduced with relative ease to a wide variety of structures, often without disruption to biological behavior of proteins. On the other hand, enzymatic ligation has been explored for selective protein modification in native linkages as well (Wu and Guo, 2012). For example, Sortase A-mediated ligation (SML) has been utilized to site-specifically modify proteins (Tsukiji and Nagamune, 2009). Specifically, Sortase A (SrtA) recognizes the unique pentapeptide LPXTG, where X is variable, of the C-terminal domain of target proteins and transfers the carboxylic group of Thr to a substrate carrying an N-terminal glycine to afford the transpeptidation products. Therefore, SML could be used for direct PEGylation of protein containing LPXTG with glycine-functionalized PEG molecule (Popp et al., 2011).

In this report, we expressed a recombinant rTM_{456}-LPETG with a FLAG fusion tag at the N-terminus and a His-tag at the C-terminus, in which the LPETG motif facilities end-point modification of rTM_{456} with diglycine chain-end functionalized PEG catalyzed by SrtA, which also has a His-tag at the C-terminus. The His-tag on both rTM_{456}-LPETG and SrtA allows for an easy cleanup of the unreacted rTM_{456}-LPETG and SrtA by nickel affinity column after the SML reaction. In our previous study, we expressed recombinant TM containing an azido-functionalized methionine analog at the C-terminus (rTM_{456}-Azide) for site-specific rTM_{456} modification (Zhang et al., 2013). However, the low expression yield of the rTM_{456}-Azide with the unnatural amino acid limits its further functional investigation and practical application as well. Alternatively, rTM_{456} containing a LPETG pentapeptide at C-terminus was expressed in higher yield, through which diGly-PEG_3-azide could be conjugated *via* SML to incorporate azide functionality into the C-terminus of rTM_{456} (Jiang et al., 2012; Wang et al., 2016). This approach affords the higher overall yield for preparing recombinant TM_{456} with azide functionality at its C-terminus and thus facilitates a site-selective PEGylation of rTM_{456} with DBCO chain-end functionalized PEG *via* Copper-free click chemistry (CFCC) (Fig. 1). Overall, both enzymatic and chemical modification aimed to add PEG to rTM_{456} at a specific site generated homogenous rTM_{456} conjugates. These site-specific modification strategies will lay the foundation for developing TM-based antithrombotic agents with enhanced antithrombotic activity and pharmacokinetic properties.

2. Materials and methods

2.1. Materials and reagents

All solvents, chemicals and reagents were purchased from commercial sources and were used unless otherwise noted. pET39b vector, pET28b vector and competent cells were purchased from EMD Chemicals. Kanamycin sulfate and IPTG (Isopropyl-beta-D-thiogalactopyranoside) were purchased from Calbiochem. The mouse monoclonal antibody specific to human TM, rabbit monoclonal antibody specific to Polyethylene glycol (PEG), goat anti-mouse IgG H&L (HRP) and goat anti-rabbit IgM mu chain (HRP) were purchased from Abcam. Human protein C, human thrombin and human antithrombin III were obtained from Haematologic Technologies Inc. Chromogenic thrombin substrate BIOHPEN CS-01(38) and bovine serum albumin (BSA) were from Fisher Scientific. Heparin, Aprotinin and Trypsin were purchased from Sigma. Pooled normal human plasma was from Innovative Research. NH_2-Gly-Gly-PEG_3-azide and NH_2-Gly-Gly-NH-PEG_{5000}-OMe were purchased from CarboSynUSA. DBCO-Cy5 and DBCO-mPEG_{5000}-OMe were purchased from Click Chemistry Tools.

2.2. Expression and purification of rTM_{456}-LPETG

The recombinant TM contained the EGF domain 4–6 of human thrombomodulin with a FLAG tag on N-terminal, a C-terminal LPETG sequence and a His tag (rTM_{456}) was expressed and purified as previously described with some modification (Jiang et al., 2012). The expression plasmid, pET39b-rTM_{456} was transformed into *E. coli.* B834 (DE3) cells for expression. The *E. coli.* B834 (DE3) cells were incubated in LB medium with 35 μg/mL kanamycin at 37 °C, shaking at 150 rpm until an OD_{600} of 0.8 was reached, and then IPTG was added to a final concentration of 1 mM to induce the overexpression of rTM_{456} by incubation for 5 h at 37 °C. The bacteria were then centrifuged at 8000*g* for 5 min, and the cell pellets were collected and purified or stored in −80 °C.

The bacteria pellets were resuspended in 10 mL of ice-cold lysis buffer (20 mM Tris and 150 mM NaCl, pH 8.0) with 1 mM PMSF (phenylmethanesulfonyl fluoride) and lysed by sonication. The extract was collected by centrifugation at 20,000*g* for 15 min, then loaded into HisTrap FF column (GE Healthcare) charged with Ni^{2+} ions and eluted with buffer (20 mM Tris, 0.5 M NaCl and 250 mM imidazole, pH 8.0). Pooled fractions containing rTM_{456}-LPETG were collected and then dialyzed by Amicon Ultra Centrifugal Filter (Millipore) with a cutoff molecular weight of 10,000 Da to afford rTM_{456}-LPETG. Sterilized glycerol was added to the rTM_{456}-LPETG solution to a final

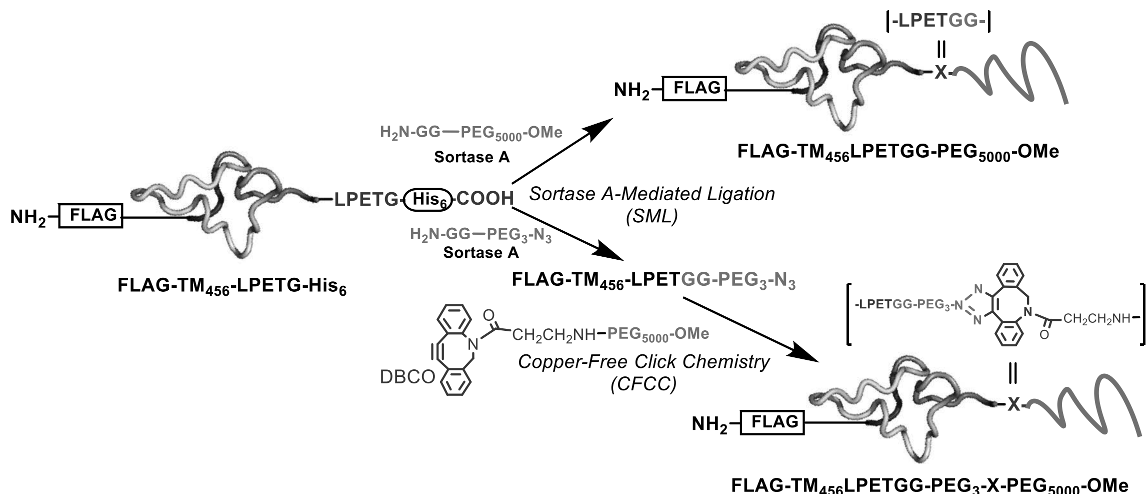

Fig. 1. Domain structures of recombinant TM_{456} fragment and its end-point PEGylation strategy *via* SML and CFCC.

concentration is 10% to keep rTM_{456}-LPETG activity. After detection of the concentration of this rTM_{456}-LPETG solution by Bradford method, the rTM_{456}-LPETG was aliquoted and stored in −80 °C.

2.3. Expression and purification of soluble SrtA

SrtA with a C-terminal His tag was expressed and purified from *E. coli* BL21 as previously described (Tsukiji and Nagamune, 2009). Briefly, the *E. coli* BL21 transformed with the plasmid pET28b-SrtA were grown in LB medium to an OD_{600} of 0.8 at 37 °C, and protein expression was induced by the addition of IPTG to a final concentration of 1 mM for 5 h. Then the bacteria cells were harvested by centrifugation and resuspended in 10 mL of ice-cold lysis buffer (20 mM Tris and 150 mM NaCl, pH 8.0) with 1 mM PMSF, and lysed by sonication. The extract was centrifuged at 20,000*g* for 15 min, and supernatant was applied onto HisTrap FF column charged with Ni^{2+} ions and eluted with buffer (20 mM Tris, 0.5 M NaCl and 250 mM imidazole, pH 8.0). The pooled fractions containing SrtA were collected and dialyzed, then kept in 10% glycerol and stored in −80 °C until use.

2.4. C-terminal covalent PEGylation of rTM_{456}-LPETG via Sortase A-mediated ligation (SML)

rTM_{456}-LPETG (14 μM) was mixed with SrtA (2 μM) and NH_2-Gly-Gly-NH-PEG_{5000}-OMe (133 μM) in reaction buffer (20 mM Tris and 150 mM NaCl, pH 8.0). The reaction mixture was then incubated at 37 °C for 3 h to obtain the C-terminal PEGylated rTM_{456} (rTM_{456}-PEG_{5000}-OMe). The obtained rTM_{456}-PEG_{5000}-OMe was purified from the remaining reaction mixture by collecting the pass-through fraction of His SpinTrap column (GE Healthcare). Excess NH_2-Gly-Gly-NH-PEG_{5000}-OMe was then removed by Amicon Ultra Centrifugal Filter (Millipore) with a cutoff molecular weight of 10,000 Da and the rTM_{456}-PEG_{5000}-OMe was concentrated. The total amount of pure rTM_{456}-PEG_{5000}-OMe was measured by Bradford assay.

2.5. Incorporation of azide functionality to the C-terminal of rTM_{456}-LPETG and its covalent PEGylation via copper-free click chemistry (CFCC)

rTM_{456}-LPETG (14 μM) was mixed with SrtA (2 μM) and NH_2-Gly-Gly-PEG_3-azide (3 mM) in reaction buffer (20 mM Tris and 150 mM NaCl, pH 8.0) to first prepare the product of rTM-PEG_3-azide. The rTM_{456}-LPETG-PEG_3-azide was purified from the remaining reaction mixture by collecting the pass-through fraction of His SpinTrap column (GE Healthcare), and dialyzed by Amicon Ultra Centrifugal Filter (Millipore) with a cutoff molecular weight of 10,000 Da to obtain the pure product. Then, the pure rTM_{456}-LPETG-PEG_3-azide (8 μM) and DBCO-PEG_{5000}-OMe (64 μM) were incubated and shaken at room temperature overnight in reaction buffer (20 mM Tris and 150 mM NaCl, pH 8.0). The excess unreacted DBCO-PEG_{5000} was removed by dialyzing *via* Amicon Ultra Centrifugal Filter (Millipore) with a cutoff molecular weight of 10,000 Da. The successful formation of rTM_{456}-PEG_3-PEG_{5000}-OMe was measured and stored in −20 °C until use.

2.6. SDS-PAGE Coomassie blue staining and Western blot analysis

The rTM_{456}-LPETG, SrtA, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were detected in 15% SDS-polyacrylamide gels under reducing conditions by Coomassie Blue staining. Also, Western blot was conducted by transferring the gel onto polyvinylidene difluoride membranes by Trans-Blot Turbo Transfer system (Bio-Rad). The membranes were blocked in 5% non-fat milk in TBST (contained 0.05% of Tween 20) for anti-TM and in 10% BSA in TBST for anti-PEG at room temperature for 1 h with gentle shaking. Primary anti-TM monoclonal antibody (1:2000) or anti-PEG monoclonal antibody (1:1000) in 5% non-fat milk or in 10% BSA was incubated with the membrane at 4 °C overnight. The membranes were then washed with TBST (3 × 10 min) and then incubated with a secondary antibody (goat anti-mouse IgG HRP or goat anti-rabbit IgM HRP 1:5000) in 5% non-fat milk for 1 h at room temperature. After washing with TBST (3 × 10 min), protein bands on the membrane were visualized using an enhanced chemiluminescence (ECL) solution (Thermo fisher).

2.7. Stability of rTM_{456}-LPETG and PEGylated rTM_{456}-LPETG conjugates under proteolytic digestion by trypsin

Briefly, 10 μg of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe or rTM_{456}-PEG_3-PEG_{5000}-OMe, was incubated with 20 μg of Trypsin in the reaction buffer (20 mM Tris and 150 mM NaCl, pH 8.0) at 37 °C for 0, 1, 2, 3, 4, 5 and 6 h, respectively. Then the reaction mixture was supplemented with SDS-PAGE reducing sample buffer, heated to 100 °C for 10 min, and then electrophoresed on SDS-PAGE (15% acrylamide) gels. The amount of undigested protein in a given lane was determined by Western blot by anti-TM and anti-PEG antibodies, respectively. Image J software was employed to calculate the band intensity of rTM_{456}-LPETG, PEGylated rTMs and their remains after Trypsin digestion in three independent experiments.

2.8. Protein C activation activity assay of rTM_{456}-LPETG and PEGylated rTM_{456}-LPETG conjugates with or without trypsin digestion

The cofactor activities of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe, rTM_{456}-PEG_3-PEG_{5000}-OMe and their Trypsin digestion products were assessed by protein C activation assay as previously described with some modification (Popp et al., 2011). Briefly, rTM_{456}-LPETG and PEGylated rTMs obtained from the above conjugation were added into assay buffer (20 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$ and 0.1% BSA, 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 while shaking, the PC activation was terminated by addition of 30 μL human antithrombin III (1 mg/mL) and 2 μL heparin (10 U/mL) for 10 min at 37 °C while shaking. The activated PC was measured by incubation with chromogenic thrombin substrate BIOHPEN CS-01(38) (0.5 mM) for 20 min at 37 °C to determine its enzymatic activity. The UV absorbance at 405 nm was measured.

For protein C activation assays, Trypsin digestion reaction of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were stopped by adding 10 μg/mL of Aprotinin in the reaction buffer to avoid the effect of Trypsin on protein C activation. All protein C activation assays were conducted as above with the presence of Aprotinin.

2.9. Thrombin clotting time (TCT) assay of PEGylated rTM_{456}-LPETG conjugates

rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe (0.002 mg/mL, 0.004 mg/mL and 0.005 mg/mL) in normal human plasma were prepared, and similar concentrations of NH_2-Gly-Gly-NH-PEG_{5000}-OMe and DBCO-PEG_{5000}-OMe were prepared as controls. The clotting of each sample (100 μL) was triggered by adding 200 μL of human thrombin (5 U/mL). The TCT was measured by BBL fibrometer (Becton-Dickinson). The initial point was obtained with buffer (20 mM Tris and 150 mM NaCl, pH 8.0) alone.

2.10. Statistical analysis

All data is presented as the means ± the standard error of the mean. Differences between experimental groups were analyzed using one-way ANOVA with an independent *t*-test. All reported p values were two-tailed, and p values of < 0.05 were considered statistically (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Statistical analyses were performed with SPSS 16.0 software.

3. Results and discussion

Translational incorporation of functional amino acids has been a very promising strategy to improve protein physicochemical and biological properties and create functional groups for site-specific protein modification applications (Boutureira and Bernardes, 2015; Hendrickson et al., 2004). Azidohomolananine can serve as a methionine surrogate and can be incorporated into proteins, providing an azidohomolananine-containing protein for chemo-selective modification (Kiick et al., 2002). In our preliminary studies, we have expressed recombinant TM_{456} construct containing an azido-functionalized methionine analog at the C-terminus for site-specific immobilization (Sun et al., 2006) and modification (Zhang et al., 2013) *via* bio-orthogonal chemistry. However, low translational efficiency of the unnatural amino acid azidohomolananine is the limiting factor for larger scale preparation, modification and functional study of the protein. Enzymatic modification provides another robust tool for site-specific protein modification (Sunbul and Yin, 2009). In our preliminary study, we expressed recombinant TM_{456} containing a LPETG pentapeptide at the C-terminus (rTM_{456}-LPETG) for enzymatic end-point immobilization (Jiang et al., 2012) and modification (Jiang et al., 2014) *via* SML. Therefore, we speculated that rTM_{456}-LPETG is versatile for site-specific PEGylation of rTM_{456} directly with glycine chain-end functionalized PEG *via* SML. In this study, the rTM_{456}-LPETG was prepared by expression in *E. coli* B834 (DE3) cells with TM_{456}GGMLPETG and all tags plasmid. After purification, the protein integrity was confirmed by SDS-PAGE (Fig. 3A). Optimization for the large-scale expression and efficient purification was conducted to provide sufficient amount of rTM_{456}-LPETG for its PEG conjugate preparation and stability and activity studies. In addition, an azide functionality was easily incorporated to rTM_{456}-LPETG with a glycine molecule containing azide *via* SML, which facilitates a site-specific PEGylation *via* CFCC.

3.1. C-terminal covalent PEGylation of rTM_{456}-LPETG via SML

It has been known that a diglycine at the N-terminus of a nucleophile shows the highest yield for Sortase-catalyzed transpeptidation and the addition of any more glycines has no significant effect on the reaction rate (Tsukiji and Nagamune, 2009). Therefore, diglycine was chosen as the nucleophile of the PEG molecule in our study. Specifically, N-terminal diglycine-containing PEG_{5000}-OMe (NH_2-Gly-Gly-NH-PEG_{5000}-OMe) was used for site-specific PEGylation of rTM_{456}-LPETG *via* SML. In this study, the His-tag at the C-terminus of rTM_{456}-LPETG and SrtA allows for an easy removal of the unreacted rTM_{456}-LPETG-His-tag and SrtA-His-tag by a nickel affinity column, while the PEGylated product was obtained directly by passing the reaction mixture through the nickel column as it does not have the His-tag due to the cleavage of T/G in LPETG motif after the SML reaction (Fig. 2). Briefly, rTM_{456}-LPETG was incubated with SrtA and NH_2-Gly-Gly-NH-PEG_{5000}-OMe in reaction buffer for 3 h at 37 °C followed by passing through the nickel affinity column to afford the C-terminal PEGylated rTM_{456}-LPETG (rTM_{456}-PEG_{5000}-OMe). The resultant rTM_{456}-PEG_{5000}-OMe was analyzed by SDS-PAGE and Western blot (Fig. 3). As shown in the SDS-PAGE gel resulting from Coomassie blue staining (Fig. 3A), a major band with larger molecular weight formed, indicating the successful PEGylation *via* SML. Also, Western blot with mouse monoclonal antibody specific to human TM (Fig. 3B) and rabbit monoclonal antibody specific to PEG molecule (Fig. 3C) confirmed the PEGylated rTM_{456}-LPETG conjugates. From the Western blot analysis with anti-TM antibody, two extra bands with much higher molecular weight were observed for the SML reaction products (Fig. 3B). The top band might be the dimer of the rTM_{456} formed during the reaction or purification step. Another one might be the rTM_{456}-LPETG-acyl-SrtA intermediate that stays unreacted with the Gly_2-PEG_{5000}-OMe nucleophile. This indicates that the larger substrate may have steric issue that prevents it reaching and reacting with the acyl-SrtA intermediate, which is in agreement with our previous finding (Jiang et al., 2014). This might be the drawback of SML with larger molecules in conjugation.

3.2. C-terminal covalent PEGylation of rTM_{456}-LPETG via CFCC

Next, we investigated an alternative PEGylation of rTM_{456} containing an azide at the C-terminus with DBCO-PEG_{5000}-OMe *via* CFCC, which is very efficient in protein modification (Jang et al., 2012). First, an azide functionality was incorporated into rTM_{456}-LPETG with diglycine-PEG_3-azide *via* SML by an efficient purification flow as shown in Fig. 2. Then, the pure rTM_{456}-PEG_3-azide was incubated with DBCO-PEG_{5000}-OMe in the same reaction buffer for 12 h at room temperature. The PEGylated rTM_{456} product was confirmed by SDS-PAGE from Coomassie blue staining (Fig. 3A) and Western blot with both anti-TM (Fig. 3B) and anti-PEG antibodies (Fig. 3C). These results demonstrate that CFCC-mediated PEGylation provides a site-specific protein modification. There is an extra band around 16 kDa observed from the CFCC-mediated PEGylation on SDS-PAGE. This may be the degraded fragment from rTM_{456} as it is unstable during 12 h long time of reaction. This extra band was not confirmed in the Western blot (Supporting Information, S1). This may be due to that the anti-TM antibody does not recognize this small fragment. Therefore, we conducted the reaction for 3 h at room temperature. As a result, there was no this extra band formed in addition to the PEGylated product (Supporting Information, S2). In addition, the unreacted rTM_{456} was observed in SDS-PAGE but not in Western blot because that only one sixth amounts of the proteins were used in Western blot. However, it was observed when larger amount of the protein was used (Supporting Information, S3).

3.3. Stability of PEGylated rTM_{456}-LPETG conjugates under proteolytic digestion by trypsin

As described above, PEGylation could enhance the protein's stability. In this study, a Trypsin digestion assay was conducted to evaluate the PEGylation effect on the stability of rTM_{456}-LPETG, which is often used to test the resistance to proteolytic degradation (Guan et al., 2004; Veronese et al., 1996). Briefly, rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe or rTM_{456}-PEG_3-PEG_{5000}-OMe was incubated with Trypsin in the reaction buffer at 37 °C for 0, 1, 2, 3, 4, 5 and 6 h, respectively. Then, all samples were electrophoresed on a SDS-PAGE gel and the undigested protein were determined by Western blot. As a result, unmodified rTM_{456}-LPETG was completely digested at 4 h, while rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were almost unchanged until 6 h after Trypsin treatment (Figs. 4B and 5A). Interestingly, both PEGylated rTM_{456}-LPETG conjugates showed an immediate breakdown at 0 h upon Trypsin treatment, which cleaved a small part from the N-terminal as

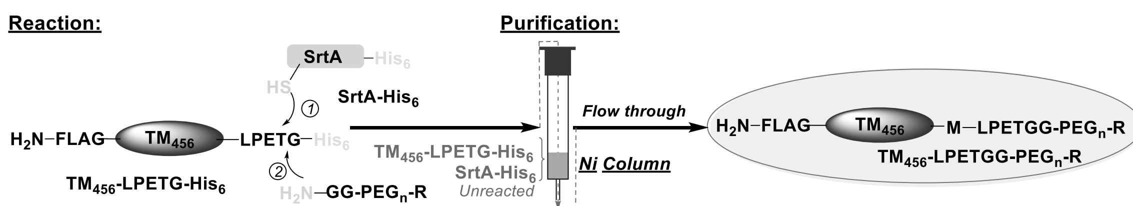

Fig. 2. Site-Specific PEGylation *via* SML and rapid purification flowchart of SML product *via* Nickel affinity column.

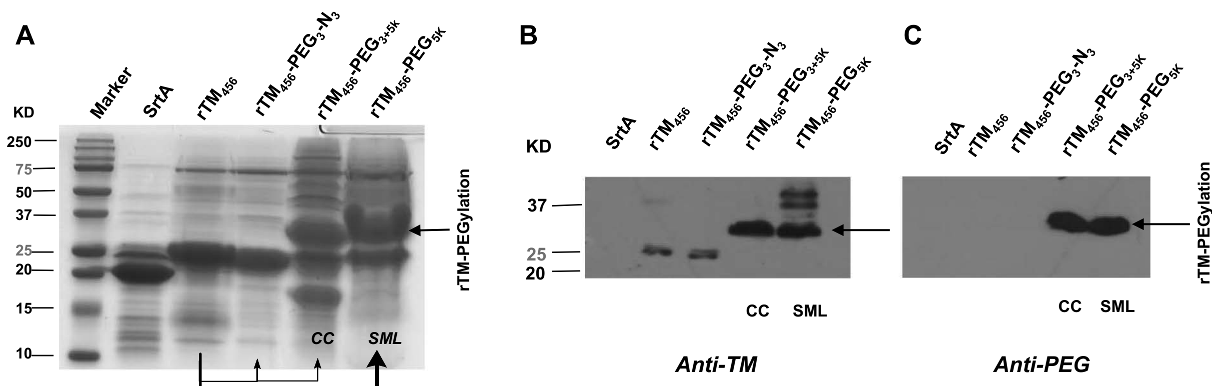

Fig. 3. Characterization of PEGylated rTM_{456} conjugates obtained from site-specific PEGylation of rTM_{456}-LPETG *via* Click Chemistry (CC) and Srtase A-mediated Ligation (SML). (A) SDS-PAGE from Coomassie blue staining and Western blot with both anti-TM antibody (B) and anti-PEG antibody (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirmed by Western blot with both anti-TM antibody and anti-PEG antibody. Both SML-mediated and CCFC-mediated PEGylation products showed the same trend of protein stability against Trypsin digestion as shown in Western blot with both anti-TM antibody (Fig. 4B) and anti-PEG antibody (Fig. 5). rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were stable in the reaction buffer (Fig. 4A). All these results indicate that PEGylation of rTM_{456}-LPETG enhanced protein stability.

3.4. Protein C activation activity assay of PEGylated rTM_{456}-LPETG conjugates with or without trypsin digestion

In order to know whether PEGylation of rTM_{456} affects rTM_{456}'s anticoagulant activity, protein C activation activities of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were measured. Briefly, rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe or rTM_{456}-PEG_3-PEG_{5000}-OMe was first incubated with thrombin and protein C to generate activated protein C (APC). Then, APC was measured by quantifying the hydrolysis product of the chromogenic substrate at 405 nm. As shown in Fig. 6A, rTM-PEG_{5000}-OMe and rTM-PEG_3-PEG_{5000}-OMe conjugates showed a similar level of protein C activation activity to rTM_{456}-LPETG, indicating that the PEGylation didn't hamper the rTM_{456}-LPETG activity. These results demonstrate that the site specific PEGylation of rTM_{456} did not affect its anticoagulant activity, even using a two-step reaction involving SML and CFCC. In addition, protein C activation capability was monitored for all Trypsin digestion sample solutions as described above. As a result, unmodified rTM_{456}-LPETG lost 50% protein C activation activity upon 4 h Trypsin digestion (Fig. 6B), while PEGylated rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe maintained protein C activation activity after 6 h Trypsin treatment (Fig. 6C and D), which are well agreement with the Western blot results above (Figs. 4 and 5). These results further confirm the

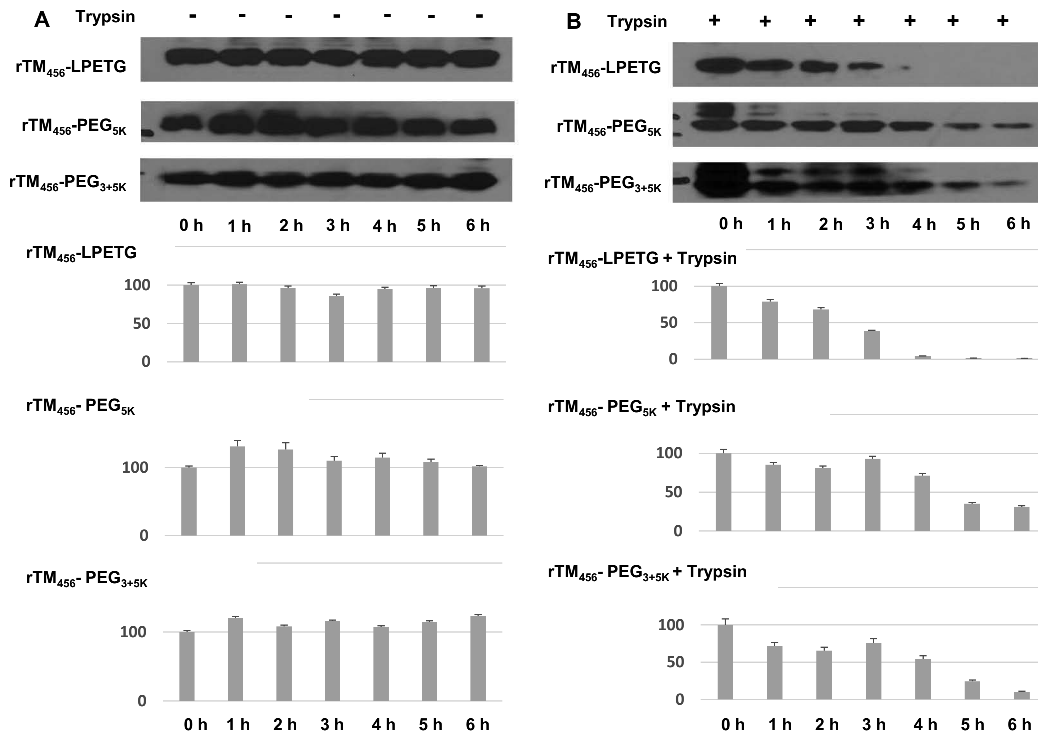

Fig. 4. Stability of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe. (A) rTM_{456}-LPETG, rTM-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe without Trypsin, (B) rTM_{456}-LPETG, rTM-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe with Trypsin examined by Western blot with mouse monoclonal anti-TM antibody. Gel intensities are normalized to 0 h time of Trypsin digestion.

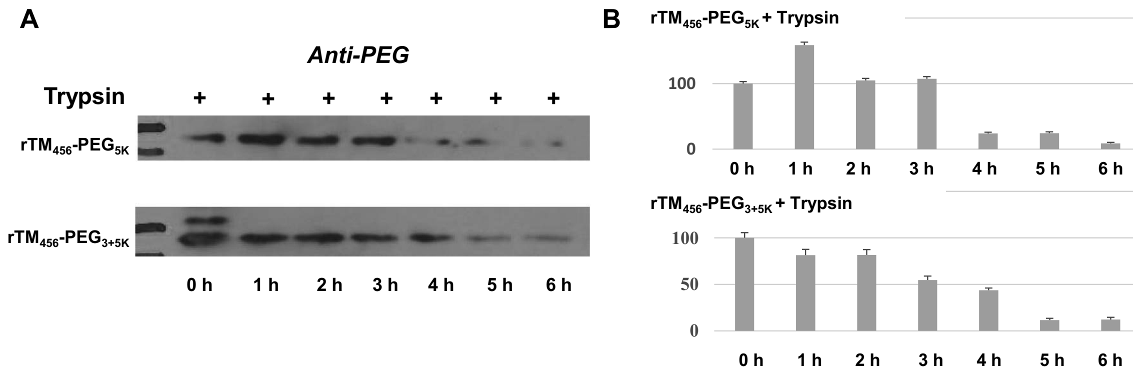

Fig. 5. Stability of rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe upon Trypsin digestion. (A) Western blot with rabbit monoclonal anti-PEG antibody, (B) gel intensity of western blot, gel intensities are normalized to 0 h time of Trypsin digestion.

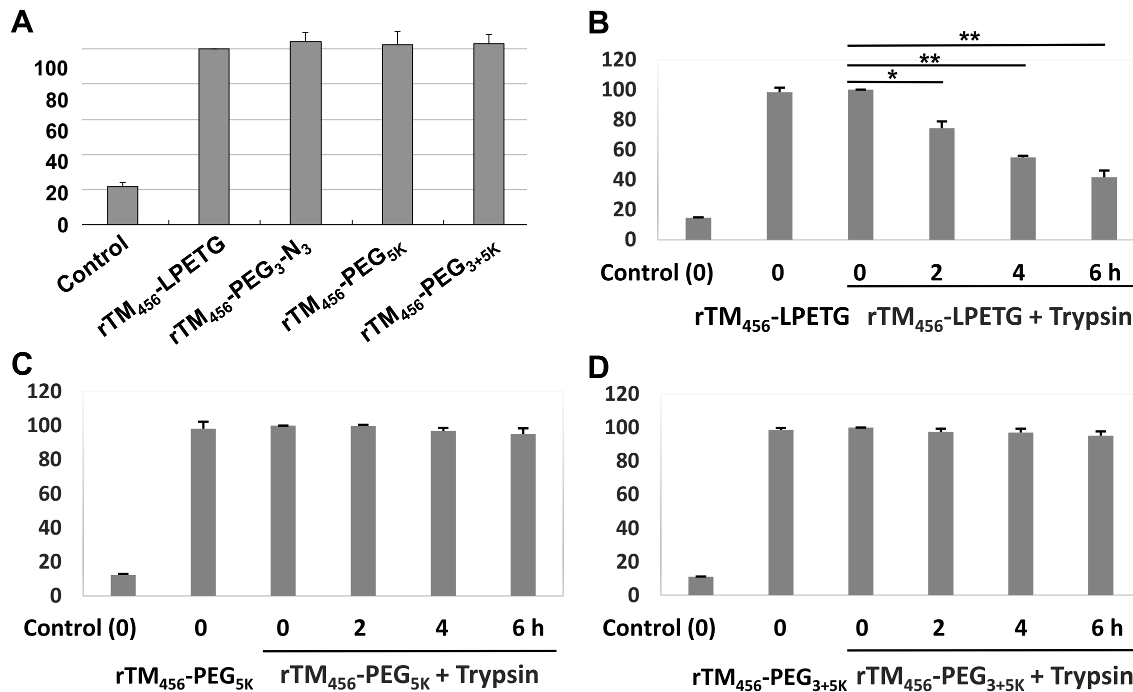

Fig. 6. Protein C activation activity. (A) rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe without Trypsin (activities are normalized to rTM_{456}), (B) rTM_{456}-LPETG, (C) rTM_{456}-PEG_{5000}-OMe and (D) rTM_{456}-PEG_3-PEG_{5000}-OMe with Trypsin (activities are normalized to 0 h time of Trypsin digestion). *p* Values of < 0.05 were considered statistically (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

enhanced stability of rTM_{456} through PEGylation.

3.5. Thrombin clotting time (TCT) assay of PEGylated rTM_{456}-LPETG conjugates

The thrombin clotting time (TCT) was measured for rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe, respectively, in order to determine the effect of PEGylation on their anticoagulation activity. Different concentrations of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe were mixed with human plasma, and then human thrombin was added to trigger the clotting. rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe showed a concentration-dependent clotting time prolongation, while the control PEG molecules did not show any activity (Fig. 7). There was a clear difference in clotting time for rTM_{456}-LPETG and PEGylated rTM_{456}-LPETG conjugates. At higher concentrations, PEGylated rTM_{456}-LPETG conjugates showed a longer TCT compared to unmodified rTM_{456}-LPETG. This result is different from the *in vitro* protein C activation assay above, in which rTM_{456}-LPETG and PEGylated rTM_{456}-LPETG conjugates showed the same level of APC generation activity. This can be explained that the TCT assay was conducted in human plasma, in which un-modified rTM_{456}-LPETG may be unstable due to enzymatic degradation. However, PEGylated rTM_{456}-LPETG may be resistant to the enzymatic degradation. This result indicates possible *in vivo* stability of the PEGylated rTM_{456}-LPETG conjugates, which is expected in a future animal study.

4. Conclusions

Protein modification warrants improved biological activity and pharmacokinetic properties. Two successful site-specific strategies for PEGylation of rTM_{456} were demonstrated *via* Sortase A-mediated ligation (SML) and copper-free click chemistry (CFCC). The PEGylation by both chemistries did not upset the rTM_{456} biological activities, such as protein C activation activity and anti-thrombin activity. Both reactions have advantages of mild reaction conditions and high yields. In

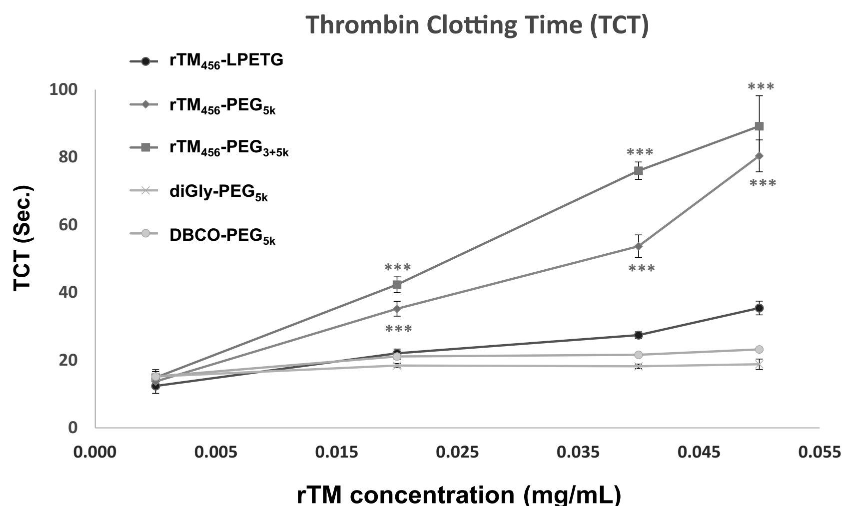

Fig. 7. Thrombin clotting time (TCT) of rTM_{456}-LPETG, rTM_{456}-PEG_{5000}-OMe and rTM_{456}-PEG_3-PEG_{5000}-OMe.

addition, the His-tag of both rTM_{456} and Sortase A allows for a quick purification of the PEGylated product *via* a nickel affinity column. We anticipate that the PEGylated rTM_{456} will show an enhanced pharmacokinetic property *in vivo*. These strategies are viable for modification of a variety of proteins with different molecules for elevated activity and stability, novel functions, and other specific properties of interest as well.

Funding

This work was supported by grants from the NIH (1R15HL138544-01, X.-L. Sun), National Science Foundation MRI Grant (CHE-1126384, X.-L. Sun) and Faculty Research Development Grant and the Research Fund from the Center for in Health and Disease (GRHD) at Cleveland State University. X. Liu appreciates the China Oversea Scholar Award from China Scholarship Council.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2019.105066.

References

Agard, N.J., Baskin, J.M., Prescher, J.A., Lo, A., Bertozzi, C.R., 2006. A comparative study of bioorthogonal reactions with azides. ACS Chem. Biol. 1, 644–648.
Boutureira, O., Bernardes, G.J., 2015. Advances in chemical protein modification. Chem. Rev. 115, 2174–2195.
Cazalis, C.S., Haller, C.A., Sease-Cargo, L., Chaikof, E.L., 2004. C-terminal site-specific PEGylation of a truncated thrombomodulin mutant with retention of full bioactivity. Bioconjug. Chem. 15, 1005–1009.
Dittman, W.A., Majerus, P.W., 1990. Structure and function of thrombomodulin: a natural anticoagulant. Blood 75, 329–336.
Dozier, J.K., Distefano, M.D., 2015. Site-specific PEGylation of therapeutic proteins. Int. J. Mol. Sci. 16, 25831–25864.
Fuentes-Prior, P., Iwanaga, Y., Huber, R., Pagila, R., Rumennik, G., Seto, M., Morser, J., Light, D.R., Bode, W., 2000. Structural basis for the anticoagulant activity of the thrombin- thrombomodulin complex. Nature 404, 518–525.
Guan, R., Sun, X.L., Hou, S., Wu, P., Chaikof, E.L., 2004. A glycopolymer chaperone for fibroblast growth factor-2. Bioconjug. Chem. 15, 145–151.
Hendrickson, T.L., de Crecy-Lagard, V., Schimmel, P., 2004. Incorporation of nonnatural amino acids into proteins. Annu. Rev. Biochem. 73, 147–176.
Itoh, S., Shirabe, K., Kohnoe, S., Sadanaga, N., Kajiyama, K., Yamagata, M., Anai, H., Harimoto, N., Ikegami, T., Yoshizumi, T., Maehara, Y., 2016. Impact of recombinant human soluble thrombomodulin for disseminated intravascular coagulation. Anticancer Res. 36, 2493–2496.
Jang, S., Sachin, K., Lee, H.J., Kim, D.W., Lee, H.S., 2012. Development of a simple method for protein conjugation by copper-free click reaction and its application to antibody-free Western blot analysis. Bioconjug. Chem. 23, 2256–2261.
Jiang, R., Weingart, J., Zhang, H., Ma, Y., Sun, X.L., 2012. End-point immobilization of recombinant thrombomodulin via sortase-mediated ligation. Bioconjug. Chem. 23, 643–649.
Jiang, R., Wang, L., Weingart, J., Sun, X.L., 2014. Chemoenzymatic bio-orthogonal chemistry for site-specific double modification of recombinant thrombomodulin. Chembiochem 15, 42–46.
Kiick, K.L., Saxon, E., Tirrell, D.A., Bertozzi, C.R., 2002. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. Proc. Natl. Acad. Sci. U. S. A. 99, 19–24.
Ma, C.Y., Chang, W.E., Shi, G.Y., Chang, B.Y., Cheng, S.E., Shih, Y.T., Wu, H.L., 2015. Recombinant thrombomodulin inhibits lipopolysaccharide-induced inflammatory response by blocking the functions of CD14. J. Immunol. 194, 1905–1915.
Nischan, N., Hackenberger, C.P., 2014. Site-specific PEGylation of proteins: recent developments. J. Org. Chem. 79, 10727–10733.
Pelegri-O'Day, E.M., Lin, E.W., Maynard, H.D., 2014. Therapeutic protein-polymer conjugates: advancing beyond PEGylation. J. Am. Chem. Soc. 136, 14323–14332.
Popp, M.W., Dougan, S.K., Chuang, T.Y., Spooner, E., Ploegh, H.L., 2011. Sortase-catalyzed transformations that improve the properties of cytokines. Proc. Natl. Acad. Sci. U. S. A. 108, 3169–3174.
Sun, X.L., Stabler, C.L., Cazalis, C.S., Chaikof, E.L., 2006. Carbohydrate and protein immobilization onto solid surfaces by sequential Diels-Alder and azide-alkyne cycloadditions. Bioconjug. Chem. 17, 52–57.
Sunbul, M., Yin, J., 2009. Site specific protein labeling by enzymatic posttranslational modification. Org. Biomol. Chem. 7, 3361–3371.
Suzuki, M., Mohri, M., Yamamoto, S., 1998. In vitro anticoagulant properties of a minimum functional fragment of human thrombomodulin and in vivo demonstration of its benefit as an anticoagulant in extracorporeal circulation using a monkey model. Thromb. Haemost. 79, 417–422.
Tsukiji, S., Nagamune, T., 2009. Sortase-mediated ligation: a gift from Gram-positive bacteria to protein engineering. Chembiochem 10, 787–798.
Turecek, P.L., Bossard, M.J., Schoetens, F., Ivens, I.A., 2016. PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. J. Pharm. Sci. 105, 460–475.
van der Poll, T., 2019. Recombinant human soluble thrombomodulin in patients with sepsis-associated coagulopathyanother negative sepsis trial? JAMA. 321, 1978–1980.
Veronese, F.M., Monfardini, C., Caliceti, P., Schiavon, O., Scrawen, M.D., Beer, D., 1996. Improvement of pharmacokinetic, immunological and stability properties of asparaginase by conjugation to linear and branched monomethoxy poly(ethylene glycol). J. Control. Release 40, 199–209.
Wang, L., Jiang, R., Sun, X.L., 2014. Recombinant thrombomodulin of different domains for pharmaceutical, biomedical, and cell transplantation applications. Med. Res. Rev. 34, 479–502.
Wang, L., Jiang, R., Wang, L., Liu, Y., Sun, X.L., 2016. Preparation of chain-end clickable recombinant protein and its bio-orthogonal modification. Bioorg. Chem. 65, 159–166.
Willems, L.I., van der Linden, W.A., Li, N., Li, K.Y., Liu, N., Hoogendoorn, S., van der Marel, G.A., Florea, B.I., Overkleeft, H.S., 2011. Bioorthogonal chemistry: applications in activity-based protein profiling. Acc. Chem. Res. 44, 718–729.
Wu, Z., Guo, Z., 2012. Sortase-mediated transpeptidation for site-specific modification of peptides, Glycopeptides, and proteins. J. Carbohydr. Chem. 31, 48–66.
Zhang, H., Weingart, J., Jiang, R., Peng, J., Wu, Q., Sun, X.L., 2013. Bio-inspired liposomal thrombomodulin conjugate through bio-orthogonal chemistry. Bioconjug. Chem. 24, 550–559.
Zushi, M., Gomi, K., Yamamoto, S., Maruyama, I., Hayashi, T., Suzuki, K., 1989. The last three consecutive epidermal growth factor-like structures of human thrombomodulin comprise the minimum functional domain for protein C-activating cofactor activity and anticoagulant activity. J. Biol. Chem. 264, 10351–10353.

Post-print standardized by MSL Academic Endeavors, the imprint of the Michael Schwartz Library at Cleveland State University, 2019