Supporting information

Targeted anti-thrombotic protein micelles

Wookhyun Kim[†], Carolyn Haller[†], Erbin Dai[†], Xiaowei Wang,[‡] Christoph E. Hagemeyer[‡], David R. Liu[#], Karlheinz Peter[‡] and Elliot L. Chaikof[†]*

[†]Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, and the Wyss Institute of Biologically Inspired Engineering of Harvard University, Boston, MA, USA

[‡]Atherothrombosis and Vascular Biology, Baker IDI Heart and Diabetes Institute, PO Box 6492, St Kilda Rd Central, Victoria 8008, Australia

[#]Howard Hughes Medical Institute, Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts 02138, USA

Submitted to *Angewandte Chemie International Edition*

[*]Address correspondence to:

Elliot L. Chaikof, M.D., Ph.D.
Department of Surgery
Beth Israel Deaconess Medical Center
110 Francis St, Suite 9F
Boston, MA 02115
Tel: (617) 632-9581
E-mail: echaikof@bidmc.harvard.edu

MATERIALS AND METHODS

All chemical reagents were purchased from either Fisher Scientific, Inc. (Pittsburgh, PA) or Sigma (St. Louis, MO) unless otherwise noted. TALON metal affinity resin was purchased from BD Biosciences, Inc. Anti-FLAG M2 affinity gel and FLAG peptide were from Sigma (St. Louis, MO). SDS-PAGE analysis and protein quantification were performed using UV and standard Bradford assay (Bio-Rad) protocols from the manufacturer.

Generation, expression and purification of GGG-diblock

The sequence of the synthetic olionucleotides encoding the triglycine and his-tag motif was as follows: Forward: 5' AATTCATTAAAGAGGAGAAATTAACTATGGGCGGTGGCC-GTGGTTCTCATCACCATCACCATCACG-3', Reverse: 5'-GATCCGTGATGGTGAT-GGTGATGAGAACCACGGCCACCGCCCATAGTTAATTTCTCCTCTTTAATG-3'. Single-stranded oligonucleotides were chemically synthesized (Integrated DNA Technologies, Inc.) and annealed to afford double stranded DNA cassettes with the EcoRI and BamHI restriction site overhangs at 5' and 3'-end, respectively. The double stranded DNA were purified, phosphorylated and ligated into the plasmid containing diblock gene to afford production of pGGG-diblock. Method used to produce the diblock gene encoding $[(VPGVG)(VPGEG)(VPGVG)(VPGEG)(VPGVG)]_{10}$ for the hydrophilic block and $[(IPGVG)_2VPGYG(IPGVG)_2]_{15}$ for the hydrophobic block, has been described previously.[1] The plasmid was transformed and propagated in the TOP10F' and purified using a plasmid spin miniprep kit (Qiagen, Inc). The diblock gene with N-terminal GGG followed by His-tag sequence was expressed in *E. coli* BL21(DE3). The cells were cultured in a sterile LB media containing 100 μg/mL ampicillin until optical density (OD_{600nm}) reached between 0.6 and 0.8 absorbance units. Production of the GGG-diblock polypeptides was induced with 1 mM IPTG at 37 ºC. After a 4 hr induction period, the cells were harvested by centrifugation at 4,500 g and 4°C for 15 min.

Purification was performed by immobilized metal affinity chromatography (IMAC) from a cell lysate under urea denaturing condition as described previously. The purified GGG-diblock was dialyzed (MWCO 3 kDa) against deionized water and lyophilized to produce a white spongy solid form. Yields for GGG-diblock were approximately 15.0 mg/L. Aqueous solutions of GGG-diblock were prepared from lyophilized specimen of purified protein polymer in distilled, deionized water at 4°C.

***MALDI-TOF mass*.** The molar mass of the diblock protein polymer was determined by MALDI-TOF MS on an ABI 4800 System in the positive linear mode. The matrix, 2-(4-hydroxyphenyl-azo)benzoic acid (HABA) was used at a concentration of 10 mg/mL in water/2-propanol (50:50). The polypeptide solution (1 mg/mL in distilled water) was mixed with the matrix solution in a ratio of 1:10 and dried under air.

Expression and purification of scFv-LPETG, TM-LPETG and eSrtA

***Generation of scFv-LPETG*.** The generation of the scFv-LPETG has been described previously.[2] The scFv-LPETG was cloned into pMT and expressed in insect cells. The purified scFv-LPETG was lyophilized and stored at -20ºC. The lyophilized scFv-LPETG was then reconstituted in cold distilled, deionized water and stored at -80°C until further use.

***Generation of TM-LPETG*.** A human thrombomodulin fragment composed of the fourth, fifth, and sixth EGF-like domains with an N-terminal FLAG tag and a C-terminal LPETG sortase substrate (TM-LPETG) was sub-cloned into the Sigma pFLAG ATS expression vector as described elsewhere.[3] The cells were cultured in a LB media containing 100 µg/mL ampicillin until optical density (OD_{600nm}) reached 0.5. Production of TM-LPETG was induced with 0.5 mM IPTG. The induced cells continued to grow for 4 hrs at 37 ºC and harvested at 4,500 g and at 4 ºC for 15 min. Cell lysis and protein extraction were performed via standard osmotic shock protocol to extract the periplasmic proteins. Cell pellets were first warmed to room temperature and resuspended in 50 mM Tris-HCl (pH 7.5), 500 mM sucrose and 1 mM EDTA and the

resuspended cells were incubated with gentle shaking for 10 minutes at RT. The cell suspension was centrifuged at 10,000 g for 10 min at 4 °C and the supernatant decanted. The cell pellet was resuspended in cold, distilled, deionized water for 10 minutes. This cell suspension was centrifuged at 30,000 g for 30 min at 4 °C. The supernatant was applied to Anti-FLAG M2 affinity chromatography and purification was performed following the manufacturer's instructions.

***Preparation of fluorescently labeled TM-LPETG*.** Lysine residues of TM were modified with amine-reactive fluorescent probe. Solutions of 40 μM TM in 5 mL of PBS were prepared at 4°C. A total of 100 μL of Texas Red NHS ester (Invitrogen) dissolved in DMSO at a concentration of 10 mM was added dropwise to the TM solutions. The reaction mixture was stirred at 4°C overnight and solutions dialyzed against PBS at 4°C for 72 hrs and passed through PD10 column to remove unreacted fluorescent dyes. The Texas Red-TM conjugate was analyzed by SDS-PAGE and visualized by illumination on a standard UV light box.

***Generation of eSrtA*.** An evolved penta mutant of sortase A (eSrtA) was previously generated by directed evolution using a yeast display system.[4] Expression and purification of eSrtA were performed as described previously. Briefly, purification was performed with immobilized metal affinity chromatography (IMAC) from a cell lysate. Elution fractions containing eSrtA were collected and dialyzed against 50 mM Tris-HCl (pH 7.5) and stored at 4°C for further use.

Preparation of GGG-micelle and sortase-mediated protein conjugation

***Preparation of GGG-micelle*.** Stock solutions of GGG-diblock were prepared by dissolving the lyophilized protein polymer (1 mg/mL) in cold pure water. For preparation of GGG-micelle, the GGG-diblock solution was diluted to 0.5 mg/mL with cold water and kept on ice for one hour. The tube containing diluted solution was subsequently transferred to a 30°C

water bath and incubated for 4 hr or overnight. A micelle suspension was stored under constant agitation at room temperature for further application.

***Sortase-mediated protein conjugation*.** Sortase reactions were performed in a reaction buffer solution of 50 mM Tris-HCl, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.5. Unless specified otherwise, all reactions were incubated for 1 hr at room temperature, and the reagents were used at the following concentrations: GGG-diblock or non-GGG-diblock, 4 - 5 μM; scFv-LPETG, 4 - 50 μM; TM-LPETG, 4 - 16 μM; and eSrtA, 1 μM. All micellar conjugates were purified via passing through size-exclusion centrifugal filters (MWCO 100kDa, EMD Millipore Corp.) to remove the unreacted LPETG-tagged proteins and eSrtA.

Characterization of protein nanomicelle conjugates

***Dynamic light scattering*.** Sample solutions were filtered through a 0.45 μm filter prior to DLS measurements that were carried out at a fixed scattering angle of 90° at 25°C. Samples were equilibrated at 25 °C for 1 min before measuring. Size distribution and polydispersity were analyzed by dynamic light scattering using a Zetasizer Nano-ZS (Malvern Instruments Ltd). Zeta potential was determined from three measurements.

***Transmission electron microscopy*.** Solutions of micellar conjugates were mixed with an equal amount of 1% PTA (phosphotungstic acid, adjusted with NaOH to pH 6.5). The mixed solution was placed onto a carbon support film on a copper grid for 5 min. Excess solution was wicked away and the grids were dried in a vacuum for 5 min. The samples were viewed with a JEOL 1400 TEM at an 80 kV accelerating voltage.

***In vitro aPC generation assay*.** TM activity was determined by incubating 50 μL of reaction mixtures, 0.7 - 1 μM of TM (equal molar concentration of TM for TM-micelle conjugates was used), 2 μM human protein C (Haematologic Technologies, Inc.), 5 mM calcium chloride, and 2 nM human α-thrombin (Haematologic Technologies, Inc.) in assay buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.5, 0.1% BSA) at 37°C for 30 min. Protein C activation by thrombin was

quenched with Antithrombin (5 nM) (Haematologic Technologies, Inc.). Activated protein C generation was determined at 405 nm using a chromogenic substrate Spectrozyme PCa (Sekisui Diagnostics LLC., Stamford, CT).

Flow cytometry. Heparinized whole mouse blood was collected via cardiac puncture under the approval of the Animal Care and Use Committee of BIDMC. Whole blood was centrifuged at 200 x g, 10 min, 23 °C to collect platelet rich plasma (PRP). Flow cytometry was performed on an LSR II (BD) with 100 µL PRP diluted 1:10 in modified Tyrode's buffer,[5] scFv (1 µg/ml) targeting was characterized to resting platelets and to 20 µM ADP-activated platelets. Secondary staining was performed using 10 µL/mL Alexa-Fluor 488 anti-His tag antibody (Qiagen). Data was analyzed using Flowjo software, all experiments were performed in triplicate.

Intravital microscopy. Surgical preparation of the mouse cremaster was performed as previously described.[6] All experiments were performed in the BIDMC Center for Hemostasis and Thrombosis Research Core. All procedures were approved by the Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. C57BL/6J mice were anesthetized with an intraperitoneal injection of ketamine HCl (125 mg/kg), xylazine (12.5 mg/kg), and atropine (0.25 mg/mL) and placed on a 37°C surgical blanket. The jugular vein was cannulated with PE 10 tubing to allow introduction of reagents, including: anti-CD42b-Dylight 649 (Emfret Analytics), scFv-micelles, TM-micelles, scFv/TM-micelles, or saline vehicle control. For co-localization experiments, His-tagged constructs were pre-incubated (10 µg/mL,10 min) with penta-His Alexa fluor 488 (Qiagen). The trachea was intubated with PE90 to facilitate breathing. The cremaster muscle was exteriorized, pinned to the stage, and superfused with thermocontrolled bicarbonate buffered saline equilibrated with 5% CO_2 in N_2. The surgical procedure was accomplished within 10 minutes. Injury to a cremaster arteriolar vessel wall was induced with a Micropoint Laser System focused through the microscope objective, parfocal

with the focal plane and tuned to 440 nm.[7] Microvessel data were obtained using an Olympus AX microscope with a 60x water immersion objective recorded with a Hamamatsu C9300-201/Gen III videoscope image intensifier interface. Coordinated image acquisition and offline data analyses were carried out using SlideBook software (Intelligent Imaging Innovations). For each treatment condition, platelet accumulation was characterized as median integrated fluorescence plotted over 3 min from n = 25 thrombi generated in 3 mice. In addition, platelet signals were quantified as area under the curve for each individual thrombus plotted against time.[7b, 8] Individual thrombi (N=25 thrombi/group) were plotted as a line graph (platelet RFU over time) AUC was determined for each individual thrombi line graph using Sigma Plot software.

REFERENCES

[1] W. Kim, J. Thevenot, E. Ibarboure, S. Lecommandoux, E. L. Chaikof, *Angew Chem Int Ed Engl* **2010**, *49*, 4257-4260.

[2] H. T. Ta, S. Prabhu, E. Leitner, F. Jia, D. von Elverfeldt, K. E. Jackson, T. Heidt, A. K. Nair, H. Pearce, C. von Zur Muhlen, X. Wang, K. Peter, C. E. Hagemeyer, *Circ Res* **2011**, *109*, 365-373.

[3] Z. Qu, V. Krishnamurthy, C. A. Haller, B. M. Dorr, U. M. Marzec, S. Hurst, M. T. Hinds, S. R. Hanson, D. R. Liu, E. L. Chaikof, *Adv Healthc Mater* **2014**, *3*, 30-35.

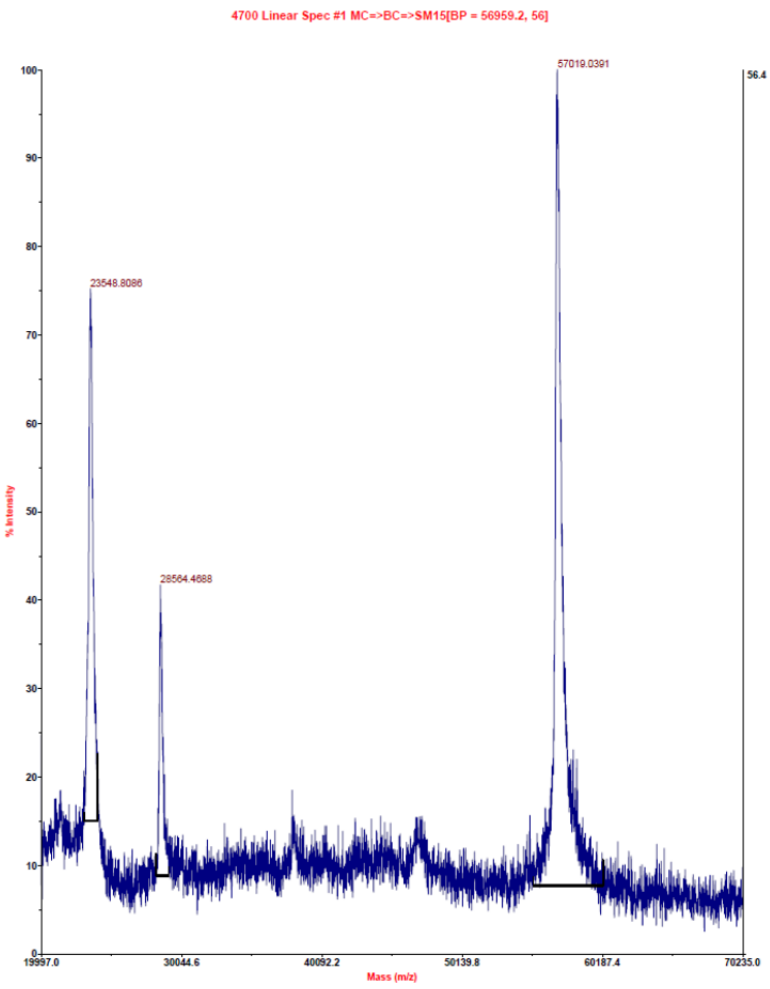
[4] I. Chen, B. M. Dorr, D. R. Liu, *Proc Natl Acad Sci U S A* **2011**, *108*, 11399-11404.

[5] L. A. Krueger, M. R. Barnard, A. L. Frelinger, 3rd, M. I. Furman, A. D. Michelson, *Curr Protoc Cytom* **2002**, *Chapter 6*, Unit 6 10.

[6] a) J. Yang, T. Hirata, K. Croce, G. Merrill-Skoloff, B. Tchernychev, E. Williams, R. Flaumenhaft, B. C. Furie, B. Furie, *J Exp Med* **1999**, *190*, 1769-1782; b) K. Ley, D. C. Bullard, M. L. Arbones, R. Bosse, D. Vestweber, T. F. Tedder, A. L. Beaudet, *J Exp Med* **1995**, *181*, 669-675.

[7] a) S. Falati, P. Gross, G. Merrill-Skoloff, B. C. Furie, B. Furie, *Nat Med* **2002**, *8*, 1175-1181; b) V. Proulle, R. A. Furie, G. Merrill-Skoloff, B. C. Furie, B. Furie, *Blood* **2014**, *124*, 611-622.

[8] R. Jasuja, F. H. Passam, D. R. Kennedy, S. H. Kim, L. van Hessem, L. Lin, S. R. Bowley, S. S. Joshi, J. R. Dilks, B. Furie, B. C. Furie, R. Flaumenhaft, *J Clin Invest* **2012**, *122*, 2104-2113.



Diblocks	MW (Da)	
	Calculated	Observed (|Δm/z|, %error)
GGG-diblock	56,751	56,969 (0.38%)
non-GGG-diblock	56,711	56,799 (0.15%)

Figure S1. MALDI-TOF mass spectra of purified GGG-diblock. Calculated and observed molecular weights of GGG-diblock and non-GGG-diblock[1] are listed below. Observed masses were averaged from three measurements.

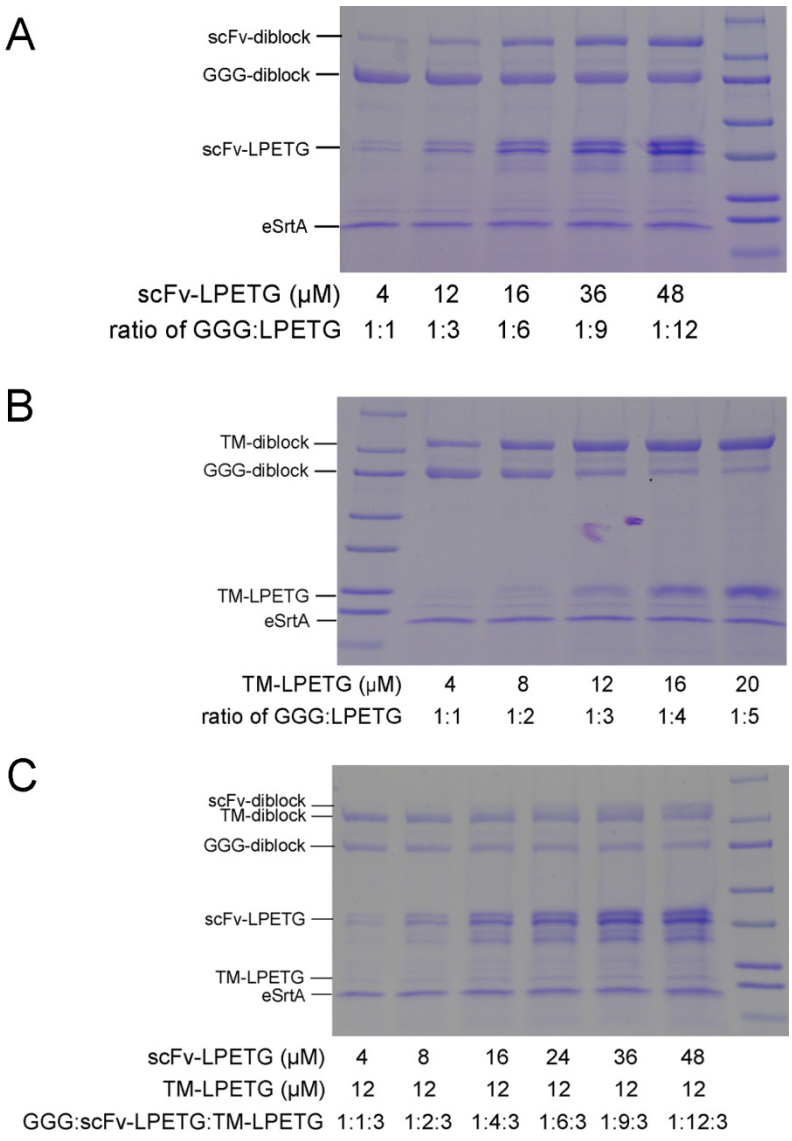


Figure S2. SDS-PAGE gels showing conjugation to GGG-diblock (4 μM) as increasing concentration of **(A)** scFv, **(B)** TM and **(C)** scFv with a constant TM concentration.

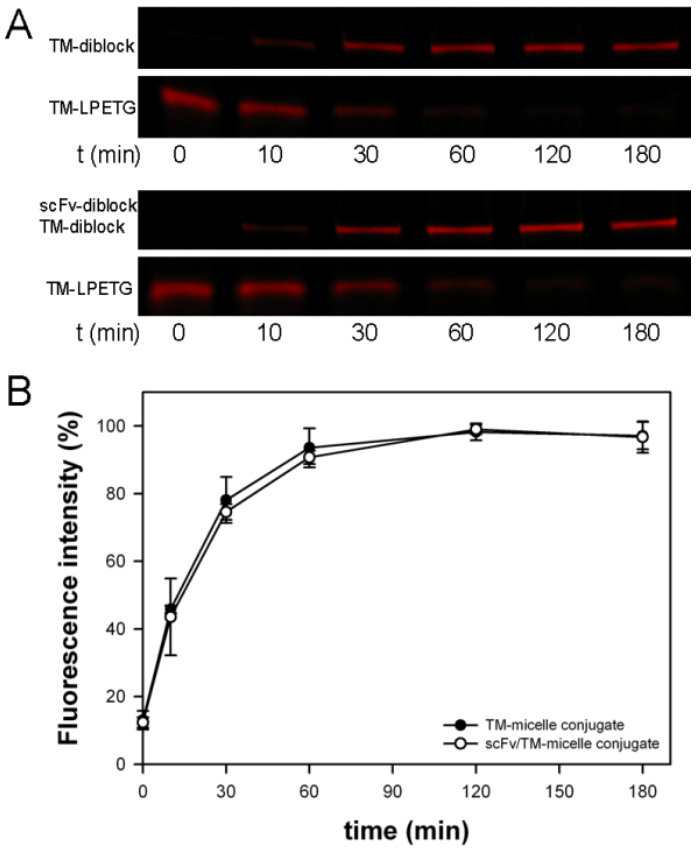


Figure S3. Time course reaction of Texas Red-labeled TM (TR-TM)-LPETG with protein nanomicelle. (A) SDS-PAGE followed by fluorescent gel imaging. **(B)** In-gel fluorescence intensities of TR-TM conjugated to GGG-diblock calculated and compared by Image J. The highest fluorescent intensity was set as 100% (n=3).

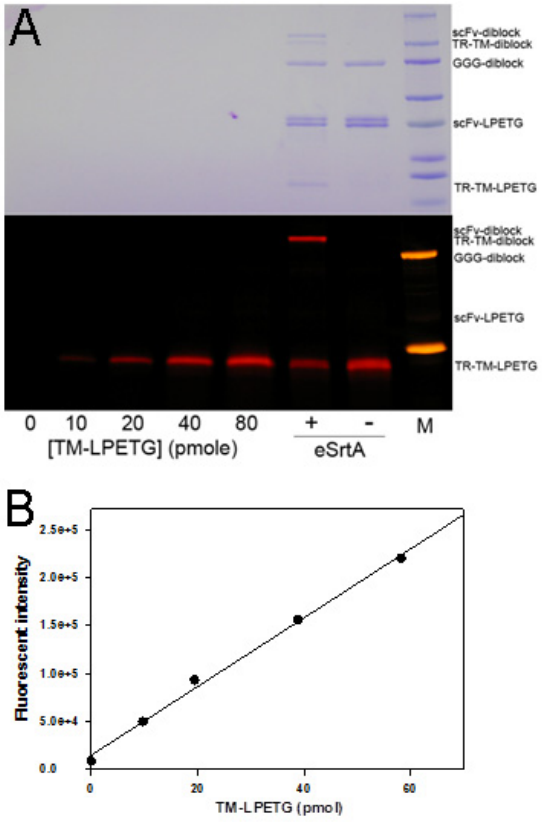


Figure S4. Quantification of TM conjugated to protein nanomicelle. TM-LPETG was labeled with Texas Red (one Texas Red per TM-LPETG). GGG-micelle was incubated with Texas Red-labeled TM-LPETG, scFv-LPETG and with or without sortase for 1 hr. **(A)** SDS-PAGE followed by fluorescence gel imagaing. **(B)** Standard curve was created by Texas Red-labeled TM-LPETG. In-gel fluorescence intensities of TR-TM conjugated to diblock calculated and compared by Image J.