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Immobilization of actively thromboresistant assemblies on sterile blood contacting surfaces

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Biomedical applications; biocompatible materials; antifouling; thrombosis; thrombomodulin

All artificial organ systems and medical devices that operate in direct contact with blood elicit activation of coagulation and platelets with long-term use,^[1, 2] leading to formation of thrombosis or emboli that often necessitates antithrombotic therapies, which carry significant cost and an attendant risk of bleeding.^[3, 4] None of the existing synthetic arterial substitutes are suitable for small-caliber (< 6 mm) revascularization procedures, which are central to the fields of cardiac, vascular, and plastic surgery, as well as for the implantation of a variety of artificial organs.^[5, 6] It is now recognized that the adverse events leading to failure of blood contacting devices is largely related to maladaptive biological reactions at the blood-material interface, which contributes to a substantial risk of early and late thrombotic occlusion.^[1, 7, 8] Despite advances in tissue engineering that have yielded biomaterials that mimic the bulk properties of native blood vessels and promote healing,^[9–11] a generally accepted blood compatible material does not exist.^[7] This work demonstrates the feasibility of a point-of-care scheme to site-specifically immobilize thrombomodulin (TM) on sterilized commercial vascular grafts, which exhibited superior thromboresistance compared with heparin-coated grafts (Gore® Propaten®) in a non-human primate model of acute graft thrombosis. Rapid one-step modification of TM with synthetic alkylamine derivatives such as azides and PEG using an evolved sortase (eSrtA) mutant may enable the industrial scaling of this approach.

Surface-induced thrombosis hinges on the generation of thrombin, a central mediator that amplifies the intrinsic coagulation cascade, crosslinks fibrin, and activates platelets.^[12, 13] First demonstrated in 1963,^[14] heparin immobilization has been hypothesized to mimic the antithrombin activity of cell surface heparan sulfate proteoglycans (HSPG) expressed by the endothelium.^[15] Recent randomized clinical trials show that heparin modified surfaces substantially improved the clinical outcomes of expanded poly(tetrafluoroethylene) (ePTFE) vascular grafts in lower extremity revascularization. Superior 1-year results have been recently reported for heparin-bonded ePTFE (Propaten) when compared to non-coated ePTFE.^[16] Preliminary analysis of heparin-bonded ePTFE covered stents in the superficial femoral artery suggests a similar effect.^[17, 18] Attenuation of thrombin production at the blood-contacting interface, therefore, may be a critical step in the design of blood-compatible materials that improve the clinical performance of blood-contacting implants.

The physiological role of heparin, produced primarily by mast cells, in hemostasis remains unclear^[19] as the majority of anticoagulant active HSPG is not in direct contact with flowing blood^[20] and knock-out mice lacking the specific pentasaccharide sequence that inhibits thrombin do not exhibit a procoagulant phenotype.^[21] In contrast, TM is the major vasculoprotective molecule localized on the endothelial cell surface and defects therein increase the risk of thromboembolism, as well as inflammatory disorders.^[22] By forming a stoichiometric 1:1 complex with thrombin, TM sequesters thrombin's prothrombotic activity, and simultaneously accelerates thrombin's catalytic activity to generate activated protein C (aPC) by 1000-fold. aPC inhibits the upstream proteases necessary for amplifying thrombin production and represents the primary physiological mechanism which regulates hemostasis.^[23, 24]

Clinical implementation of strategies reported to date on the immobilization of TM,^[25–31] as well as other proteins in therapeutic and diagnostic applications, have been primarily limited by two main factors: the inherent reduction in activity associated with schemes that non-

specifically react with free amino, carboxyl, or thiol motifs contained near the catalytically active site, and the necessity for terminal sterilization, which in the medical device industry mainly comprise either ethylene oxide (EtO) or radiation that often perturb the chemical structure and activity of surface-bound biomolecules.^[32–34] We were first to demonstrate a bioorthogonal strategy to site-specifically tether recombinant human TM via a single C-terminal azide motif (TM-N₃) by Staudinger Ligation on the luminal surface of 4 mm i.d. ePTFE grafts that preserved the therapeutic activity of TM to catalyze aPC generation.^[35] In principle, immobilization of TM on sterilized surfaces with stable anchor sites may provide an alternative to terminal sterilization to manufacture an off-the-shelf product. However, the limited yield and scalability of auxotrophic incorporation of azido-alanine in TM-N₃ as well as the propensity of triphenylphosphines to degrade by air oxidation^[36] in our previous report would prohibit the clinical translation of such an approach.

SrtA is a calcium-dependent cysteine transpeptidase found in *Staphylococcus aureus* and other Gram-positive bacteria that anchors proteins containing a C-terminal LPXTG motif (where X denotes any amino acid) by breaking the threonine-glycine bond and forming a new amide bond with oligoglycine-linked molecules.^[37–39] Site-specific SrtA transpeptidation of synthetic nucleophiles may be a more efficient alternative to unnatural amino acid approaches^[40] to incorporate any of a number of functionalities, such as biotin, fluorophores, and azides, to the termini of recombinant proteins in one step.^[41, 42] We generated a recombinant human TM fragment (Figure S1, Supporting Information) containing the minimal functional domains for antithrombotic activity^[43, 44] and a C-terminal LPETG sequence (TM_{LPETG}) that facilitated subsequent C-terminal fusion with a biotinylated pentaglycine peptide probe (Gly₅-biotin) and wild-type (WT) SrtA (Figure S2, Supporting Information). TM_{LPETG} exhibited similar catalytic activity to generate aPC in the presence of thrombin and calcium compared with previous batches of TM generated in our lab.^[44] However, high molar excess of WT SrtA (> 2 molar equiv) and Gly₅-biotin (> 10 molar equiv), as well as long reaction times (20 h), were required to drive TM_{LPETG} biotinylation yields (Figure S3, Supplementary Information). Our observations are in agreement with other reported SrtA-catalyzed protein labeling applications, which were similarly limited by the low catalytic activity of WT SrtA.^[41, 42] In addition, we confirmed previous reports showing the suitability of alkylamine nucleophile substrates for SrtA^[42, 45] by demonstrating WT SrtA-catalyzed PEGylation of TM_{LPETG} in the presence of 100 molar equiv of 5 kDa NH₂-PEG, which achieved ~80% yield after 20 h (Figure S4, Supporting Information). Despite their lower efficiency as nucleophiles, the relative synthetic simplicity of alkylamine compared with oligoglycine derivatives may further improve the commercial scalability and scope of suitable substrates for SrtA-catalyzed protein labeling.

The recent development of a general yeast display system has facilitated directed evolution of WT SrtA to generate a SrtA pentamutant (eSrtA), which exhibited 120-fold increase in catalytic activity,^[46] and enabled the routine preparation of recombinant proteins that contain a single C-terminal α -thioester.^[47] We hypothesized that eSrtA may facilitate rapid labeling of TM_{LPETG} with a single azide motif using the commercially available alkylamine derivative NH₂-PEG₃-N₃ to generate TM-N₃ (Figure 1a). As shown in Figure 2a, eSrtA dramatically improved the PEGylation efficiency of TM_{LPETG} with 100 molar equiv of 5 kDa NH₂-PEG probe as compared to WT SrtA, achieving nearly 80% yield after 2 h with 0.1 molar equiv eSrtA. This rapid one-step reaction may be broadly applied to site-specifically PEGylate LPETG-tagged recombinant protein therapeutics, which has been routinely performed to optimize half-life and biostability.^[48, 49] Notably, the reaction of TM_{LPETG} with 1 or 10 molar equiv 5 kDa NH₂-PEG for 2 h yielded minimal TM-PEG product, and no major TM multimer products formed at these conditions (Figure S5). These observations indicate minimal non-specific transpeptidation with exposed primary amines on TM_{LPETG} despite the enhanced catalytic activity of eSrtA. eSrtA was equally efficient in

catalyzing the biotinylation of TM_{LPETG} (Figure 2b) in the presence of 100 molar equiv Gly₅-biotin and NH₂-PEG₃-biotin, demonstrating equivalency of alkylamines as compared to oligoglycines for eSrtA transpeptidation when reacted in high molar excess. Under optimal reaction conditions, 0.1 equiv eSrtA and 100-fold molar excess NH₂-PEG₃-N₃ relative to TM_{LPETG} yielded a TM-N₃ conjugate in 2 h, which exhibited selective reactivity with alkyne probes (Figure 2c). As the high excess of alkylamine derivatives could be removed or recovered by established dialysis methods due to their small molecular weight, and His-tagged eSrtA could be efficiently removed by applying the crude reaction mixture onto a metal-affinity column (Figure S6, Supporting Information), commercial scale-up of TM-N₃ production by eSrtA transpeptidation should be feasible.

Optimization of strained alkynes has yielded variants that exhibit substantially higher reaction kinetics than Staudinger Ligation, while maintaining bioorthogonality to undergo cycloaddition with azide without copper catalysis.^[50, 51] Dibenzocyclooctyne (DIBO) has been among the most well characterized strained alkynes and has been commercialized for cell imaging applications.^[52] We synthesized a DIBO-PEG₁₁-NH₂ linker that enabled the activation of the luminal surface of 4 mm i.d. ePTFE vascular grafts, as well as solvent-cast polyurethane (PU) films, with DIBO anchor groups using an approach reported previously^[35] and outlined in Figure 1b. Commercial ePTFE grafts activated with DIBO reacted specifically with Rh-N₃, as verified visually (Figure S7, Supporting Information). Significantly, we demonstrated that EtO sterilization of DIBO modified surfaces did not impair their capacity to bind azide derivatives. Figure 3a shows the capacity of DIBO to specifically react with rhodamine azide (Rh-N₃) was not diminished following EtO sterilization of DIBO modified PU films. Quantitative measurement of surface biotin density in Figure 3b confirmed no significant change in DIBO reactivity with azide-PEG₃-biotin following EtO sterilization of DIBO modified ePTFE grafts. Covalent immobilization of TM-N₃ on EtO sterilized ePTFE grafts modified with DIBO maintained similar levels of catalytic activity to generate ~15 ng aPC cm⁻² min⁻¹ as TM-N₃ immobilized on non-sterile DIBO modified grafts. Collectively, these results support the feasibility of immobilizing TM-N₃ on sterilized DIBO-modified surfaces that would be carried out in the operating room prior to implantation. Similar point-of-care approaches have been described, for example, electrospinning of polymer wraps for autologous vein grafts,^[53] as well as endothelial cell seeding of ePTFE grafts^[54] prior to implantation, though these methods may carry a higher risk of microbial contamination.

Due to the lack of clinical data that establish the minimal aPC flux required to reduce risk of graft thrombosis, the therapeutic characteristics of TM modified ePTFE grafts was evaluated in an animal model. Chronic arteriovenous shunt models in baboons, whose hemostatic systems most closely mirror that of man, are routinely used to assess the thrombogenicity of blood contacting materials under dynamic flow *in vivo*.^[55–57] Due to tissue injury at the graft anastomosis, which results in exposure of highly thrombogenic tissue factor and collagen motifs to flowing blood,^[13] we devised a multi-compartment shunt configuration that inserted an upstream Dacron graft segment that serves as a thrombogenic stress to test the therapeutic activity of aPC generated in a downstream TM modified graft segment (Figure 4a). We postulated that the Dacron segment may provide a more clinically relevant test bed by reconstituting the *in vivo* thrombogenic stress exerted on implanted graft materials. Thrombogenicity of materials was evaluated by measuring the real-time deposition of radio-labeled autologous platelets on the luminal surface of unmodified ePTFE, TM modified ePTFE, as well as commercial heparin modified ePTFE (Propaten) vascular grafts.

Dacron was prone to platelet deposition as evident by steady accumulation over a 1 h perfusion period (Figure 4b), which was similar to previous reports.^[58, 59] TM modified

ePTFE graft segments reduced platelet deposition by ~80% (Figure 4c) when compared to unmodified grafts ($n=5$, $p < 0.01$). The therapeutic efficacy of TM was significantly greater than the commercial heparin coating, which resulted in a ~50% reduction in platelet deposition relative to unmodified grafts ($n=5$, $p < 0.05$). Minimal deposition of platelets on heparin modified ePTFE in non-human primates had previously been reported in shunt configurations that lacked the upstream Dacron segment,^[60] but our current studies suggest that the antithrombin III activity catalyzed by heparin may be overwhelmed in the presence of a high upstream thrombogenic source. Given the many factors that dictate the durability of an arterial substitute, the benefit afforded by TM immobilization will need to be evaluated in long-term surgically implanted bypass configurations *in vivo*.^[61]

In summary, we have demonstrated the feasibility of covalently immobilizing TM-N₃ on EtO sterilized ePTFE grafts as a point-of-care delivery scheme, which may be a key step in the clinical translation of biologically active films for permanent implants as a sterile off-the-shelf product. Significantly, we demonstrated the superior blood-contacting properties of TM-modified vascular grafts as compared to current commercial heparin modified grafts in a primate model of acute graft thrombosis. Moreover, we have optimized the scalability of producing TM-N₃ by rapid one-step eSrtA transpeptidation of alkylamine azide, and demonstrated the suitability of this approach to site-specifically PEGylate or biotinylate TM. Based on growing reports to date on the application of WT SrtA, the enhanced reactivity of eSrtA may be a key enabling step in the industrial scaling of these strategies.

Experimental Section

Materials

All reagents were purchased from Aldrich, St Louis and used without further purification unless otherwise noted. Expression and purification of recombinant TM_{LPETG} and sortases, as well as solution phase sortase transpeptidation reactions are detailed in the supplementary information section. Gly₅-biotin was obtained from Genscript, Piscataway and used without further purification.

Synthesis of DIBO-PEG₁₁-NH₂

The activated DIBO was synthesized according to a previously published protocol.^[52] DIBO-PEG₁₁-NH₂ was synthesized by reaction between the activated DIBO and t-Boc-N-amido-dPEG11-amine (Quanta BioDesign, Powell) followed by TFA deprotection, as detailed previously.^[62]

Modification of ePTFE Grafts

5% w/v Elasthane 80A (Polymer Tech, Berkeley) in *N,N*-dimethylformamide (DMF) was extruded through 4 mm i.d. ePTFE thin wall vascular grafts (Bard, Tempe) and dried under vacuum at 60 °C for 20 min. Additional PU was deposited in multiple flow-through/drying cycles over the base layer, and dried overnight under vacuum at 60 °C. PU coated grafts were inserted into tubular rotary reactors, as described previously for surface reactions.^[35] Grafts were first reacted with HDI (16% v/v) and TEA (4% v/v) in toluene at 50 °C for 1 h and rinsed in toluene for 6 h. Isocyanate activated grafts were reacted with DIBO-PEG₁₁-NH₂ linker (5 mg/mL) and DMSO (1% v/v) in toluene at 40 °C overnight, rinsed with toluene for 6 h, and dried under vacuum at 25 °C overnight. Standard EtO sterilization was carried out at the Beth Israel Deaconess Medical Center Central Processing (Boston MA). DIBO activated grafts were reacted with 1 mg/mL tetramethylrhodamine-5-carbonyl azide (Invitrogen, Carlsbad) in 1:4 tert-butanol/TBS at 37 °C for 24 h followed by rinsing in methanol for 2 days, 1 mM biotin-PEG₃-N₃ (Pierce, Rockford) in TBS at 37 °C for 24 h followed by rinsing in TBS for 24 h, or 20 μM TM-N₃ in Tris buffered saline (TBS, 20mM

Tris, 100mM NaCl, pH 7.5) at 37 °C for 24 h and rinsed with TBS for 24 h. Grafts coated with PU and activated with DIBO were reacted with rhodamine B or TM_{LPETG} as controls.

Modification of polyurethane films

Thin clear PU films were solvent cast by drying 12.5% w/v PU in DMF for 24 h at 60 °C under vacuum. Films were subjected to isocyanate, DIBO, and rhodamine reactions in a glass vial using identical conditions as graft substrates, and characterized by UV-Vis spectroscopy (Cary 50 Bio UV-visible spectrophotometer, Varian).

TM graft cofactor activity assay

A 4 mm graft segment was incubated in 200 µL of 0.2 µM human protein C (Calbiochem, Gibbstown), 5 mM calcium chloride, and 2 nM human α-thrombin (Haematologic Technologies, Essex Junction) in Tris buffer (20 mM Tris, 100 mM NaCl, pH 7.5) with 0.1% bovine serum albumin (BSA) at 37 °C for 1 h, and quenched with 2 IU/mL human anti-thrombin III (American Diagnostica, Stamford) for 5 min. The generation of aPC was quantified using 0.2 mM of an enzymatically digestible chromogenic substrate, Spectrozyme PCa (American Diagnostica).

Determination of biotin surface density with streptavidin-HRP probe

All assay reactions were run in a 96-well plate, per manufacturer's instructions (Pierce). Moles of biotin motifs bound to graft samples were determined using a standard curve generated by known concentrations of streptavidin-HRP incubated with TMB substrate. At the end of each assay, graft segments were flattened between glass slides and scanned at 600 dpi using a HP ScanJet 5370C scanner and the reactive area of each test sample was measured using ImageJ software.

In vivo baboon arteriovenous shunt model

Chronic exteriorized Silastic tubings were implanted in male baboons, as described previously.^[63, 64] All studies were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University. Mean blood flow rate through the shunt was measured continuously using a Doppler ultrasonic flow meter and held constant by an external screw clamp at 100 mL/min. Autologous platelets were radiolabeled one day prior to shunt study with Indium-111 oxine and re-injected into the baboon. Platelet deposition in the individual compartments of the shunt assembly was measured over a 60-minute perfusion period using a high sensitivity 99Tc collimator and scintillation camera (GE 400T, General Electric) imaging of the 172 keV ¹¹¹In photon peak at 5-min intervals. Thrombogenic devices were assembled and inserted into shunts, as detailed previously.^[35]

Statistical Analysis

Two-tailed student's t-test assuming unequal variances was used to test for statistical significance between the means of two groups.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) Covalent site-specific ligation of amine-PEG_n-X to thrombomodulin (TM) containing a C-terminal LPETG peptide motif catalyzed by an evolved *S. aureus* sortase A mutant (eSrtA), where n is the number of PEG repeats and X is a bioorthogonal reactive group such as biotin and azide. (b) Surface reaction scheme to modify the lumen of expanded poly(tetrafluoroethylene) (ePTFE) vascular grafts with dibenzocyclooctyne (DIBO) that can be sterilized by ethylene oxide (EtO) prior to strain-promoted [3+2] cycloaddition to immobilize azide derivatives such as rhodamine, biotin, and TM.

Figure 2.

(a) SDS-PAGE gel tracking of evolved (eSrtA) or wild-type sortase A (WT SrtA) catalyzed transpeptidation of an amine-PEG₁₁₃ (MW 5kDa) to thrombomodulin (TM) containing a C-terminal LPETG peptide motif (TM_{LPETG}) over a 16 h time period. (b) Western blot comparison of eSrtA-catalyzed transpeptidation of amine-PEG₃-biotin (NH₂-PEG₃-Bio) and biotinylated pentaglycine peptide (NH₂-Gly₅-Bio) under identical reaction conditions. (c) Strain-promoted [3+2] cycloaddition of dibenzocyclooctyne-biotin (DIBO-Bio) with azide-tagged TM, generated by eSrtA-catalyzed ligation of NH₂-PEG₃-N₃ to TM_{LPETG}.

Figure 3.

(a) UV-vis spectroscopy of rhodamine-azide (Rh-N₃) or rhodamine B (Rh) immobilized on ethylene oxide (EtO) sterilized and non-sterilized thin polyurethane (PU) films functionalized with dibenzocyclooctyne (DIBO). (b) Surface density of biotin-PEG₃-N₃ immobilized on EtO sterilized and non-sterilized expanded poly(tetrafluoroethylene) (ePTFE) grafts modified with DIBO. (c) Activated protein C (aPC) production by EtO treated and non-treated ePTFE grafts.

Figure 4.

(a) Baboon arteriovenous shunt model to measure deposition of platelets on modified expanded poly(tetrafluoroethylene) (ePTFE) grafts in the presence of an upstream prothrombotic Dacron segment. (b) Quantity of platelets deposited over a 1 h period on the Dacron segment. (c) Quantity of platelets deposited over a 1 h period on unmodified ePTFE control grafts, heparin modified Propaten grafts, and TM modified grafts (* p < 0.05, ** p < 0.01).