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Bioactive Anti-thrombotic Modification of Decellularized Matrix for Vascular Applications

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Abstract

The decellularized matrix derived from porcine small intestinal submucosa (SIS) is a widely-used biomaterial being investigated for numerous applications. Currently, thrombus deposition and neointimal hyperplasia have limited the use of SIS in some vascular applications. To limit these detrimental processes, this work applies bioactive, endothelial-inspired properties to the material. SIS was modified with the endothelial cell membrane protein thrombomodulin and the glycosaminoglycan heparin to facilitate protein C activation and anticoagulant activity, respectively. Modifying SIS with thrombomodulin alone enabled robust APC generation, and thrombomodulin activity was maintained after prolonged exposure to fluid shear and blood plasma. Heparin-modified SIS had a potent anticoagulant activity. When both modifications were applied sequentially, SIS modified first with thrombomodulin then with heparin retained the full activity of each individual modification. Tubular SIS devices were connected to a baboon arteriovenous shunt to quantify thrombus deposition on these materials. After being exposed to flowing whole blood for 60 minutes, SIS devices modified first with thrombomodulin then with heparin had significantly less platelet accumulation compared to unmodified SIS devices. These studies demonstrate that modifying SIS with thrombomodulin and heparin confers APC generation and anticoagulant activity that results in reduced thrombogenesis.

Keywords

Decellularized biomaterial; Thrombomodulin; Heparin; Coagulation; Platelets

1. Introduction

Decellularized extracellular matrices have been widely used as biomaterial scaffolds for reconstructing tissues due to their topographical and biochemical cues that help coordinate proper cellular function. One decellularized matrix widely used for soft tissue repair is derived from porcine small intestinal submucosa (SIS).[1] In addition to soft tissue repair, SIS has been utilized in cardiovascular applications including vascular grafting,[2]–[5] stent coverings,[6] pulmonary valve reconstruction,[7][8] and venous valve replacement.[9][10] In these blood-contacting applications, the primary function of the SIS is to serve as a non-

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thrombogenic surface while also facilitating endothelialization, as the development of a confluent endothelium enables long-term thromboprotection.[11] In some of these applications, for example a large diameter vascular graft implanted in the infrarenal aorta of dogs, SIS facilitated endothelialization and remodeling while remaining patent.[2][3] However, in applications more prone to thrombosis and endothelial cell dysfunction, such as venous valve prostheses, thrombus formation and neointimal ingrowth on SIS inhibited valve function.[10] Strategies to rapidly endothelialize SIS leaflets, either through seeding prior to implantation or by using immobilized capture antibodies to bind circulating endothelial progenitor cells, did not show significant reduction in this detrimental remodeling.[12] Thus, alternative strategies to reduce thrombus formation and intimal hyperplasia on SIS are needed to broaden the utility of SIS as a vascular biomaterial.

Blood-contacting materials have traditionally been designed to be inert with regard to the activation of blood components, such as platelets and coagulation factors, by minimizing the adsorption of circulating cells and proteins. Examples of these materials include expanded polytetrafluoroethylene (ePTFE) and Dacron, which are widely used in vascular grafts. However, due to the limited protein and cell adhesion on these materials, as well as their non-biodegradable composition, there is little to no endothelialization that occurs post-implantation. In contrast, the SIS biomaterial is composed of multiple proteins and growth factors that encourage integration with the surrounding tissue. Thus, strategies that limit thrombus formation and promote endothelialization on SIS must be fundamentally different than traditional synthetic materials to preserve the biologically-active properties of SIS.

Endothelial cells line the luminal surface of blood vessels and regulate multiple processes including plasma coagulation, platelet adhesion, and smooth muscle cell proliferation. Much of this regulation is facilitated by molecules expressed on the luminal surface of endothelial cells. This work applies bioactive modifications to SIS that are inspired by the endothelial cell surface to recapitulate specific endothelial functions. In particular, modifications that either catalyze the generation of activated protein C (APC), an anti-coagulant and cytoprotective enzyme, or enhance the anticoagulant activity of antithrombin III (ATIII), were selected as APC and ATIII have well-characterized mechanisms that limit both thrombosis and intimal hyperplasia (Figure 1).

Thrombus formation on blood-contacting materials is caused by both the accumulation and activation of platelets as well as the initiation of plasma coagulation cascades that generate thrombin near the blood-material interface. Thrombin is the central enzyme in coagulation and promotes thrombus formation by activating platelets and cleaving fibrinogen to generate a fibrin thrombus. To counteract these processes, the vascular endothelial cell surface protein thrombomodulin binds circulating thrombin and blocks the binding of fibrinogen while simultaneously acting as a cofactor to enhance APC generation by thrombin.[13][14] APC functions as an anticoagulant by proteolytically degrading coagulation factors necessary for the prothrombin to thrombin conversion, as well as inhibiting the intrinsic coagulation pathway, which is necessary for accelerating thrombin generation to a concentration needed for thrombosis.[15][16] In addition to anticoagulant effects, APC attenuates thrombin- and inflammation-induced endothelial apoptosis to maintain endothelium-dependent regulation

of thrombosis and vascular smooth muscle proliferation.[17][18] Thus, thrombomodulin and APC act through multiple pathways to limit thrombosis and intimal hyperplasia.

The luminal surface of endothelial cells is covered with a glycosaminoglycan-rich layer known as the glycocalyx that reduces blood cell adhesion and enhances the activity of anticoagulant molecules such as ATIII.[19] Heparin is a glycosaminoglycan that markedly increases ATIII activity by accelerating the irreversible inhibition of Factor Xa (FXa) and thrombin. Unfractionated heparin enhances ATIII activity through two mechanisms.[20] A pentasaccharide sequence of heparin binds to ATIII, changing ATIII conformation to accelerate inhibition primarily of FXa. Long heparin molecules also act through a template mechanism by which both ATIII and activated coagulation factors bind the same negatively-charged heparin molecule to increase their rate of interaction.[21] Modifying materials with heparin has been applied to metal stents and vascular graft materials to reduce thrombosis.[22][23] However, these modifications have not been applied to SIS, and the interaction of SIS modified with both thrombomodulin and heparin is unknown. Therefore, this work characterizes the effect of modifying SIS with thrombomodulin and heparin on APC generation, blood plasma coagulation and thrombus formation to advance the use of modified SIS as a biomaterial for vascular applications.

2. Materials and Methods

2.1 Reagents

Vacuum-pressed SIS was generously provided by Cook Biotech. Thrombomodulin, protein C, α-thrombin, and APC were from Haematologic Technologies. Heparin sodium solution was from Sigma Aldrich. Baboon platelet poor plasma from at least 5 animals was pooled using previously collected aliquots that had been stored at −80°C. The prothrombin time (PT) reagent Innovin® was purchased from Dade®, and the activated partial thromboplastin time (APTT) reagent HemosIL® was from Instrumentation Laboratories. The chromogenic substrate S-2366 used for APC quantification was from Chromogenix. Baboon carotid endothelial cells (ECs) were isolated as described previously [24][25]. Baboon ECs were cultured in endothelial growth medium-2 (EGM-2) from Lonza, supplemented with the MV BulletKit and 10% HyClone™ fetal bovine serum (FBS, GE Healthcare Life Sciences). Phosphate buffered saline (PBS) with Ca^{2+} and Mg^{2+} was from Mediatech, Inc.

2.2 Modification of SIS

Solutions of thrombomodulin and of heparin were diluted in tris-buffered saline (TBS; 50 mM Tris-Cl, 150 mM NaCl, pH 7.5). Unless otherwise noted, SIS was cut into 5 mm discs using a biopsy punch and placed into wells of a 96-well plate. SIS was modified by soaking in thrombomodulin or heparin solutions for 2 hrs at room temperature. Adsorption was chosen as the surface modification strategy because prior work demonstrated that carbodiimide-mediated crosslinking of SIS reduced the hemocompatibility.[26] Furthermore, permanent attachment of heparin and TM was not considered necessary because even short-term administration of either thrombomodulin[27] or APC[28] has shown a significant reduction in long-term intimal hyperplasia, and multiple inhibitory blood plasma proteins would eventually degrade the activity of both modifications.[21]

2.3 Baboon Carotid Endothelial Cell Culture

Endothelial cells (ECs) were isolated from baboon carotid arteries as previously described [29]. Briefly, pre-warmed collagenase type II (Worthington Biochemical, 600 U/mL) was dripped into the lumens of explanted carotid arteries for 5 minutes, then arteries were manually compressed to detach ECs from the vessel wall. The cell solution was then dripped into well plates coated with 50 μg/mL collagen I. Cells were cultured in EGM-2 media supplemented with 10% FBS. When confluent, cells were passaged with TrypLE (Life Technologies).

2.4 APC Generation

Following modification of SIS, punches were washed 3 times in PBS with Ca^{2+} and Mg^{2+}, followed by a 1 hr reaction in a solution of protein C (100 nM) and thrombin (5 nM) at 37°C. After the reaction, two replicates per sample were removed and placed into wells containing the direct thrombin inhibitor hirudin (final conc. 1 μM) to quench the reaction. The APC concentration was determined by adding the chromogenic substrate S-2366 and a plate reader (Tecan Infinite m200) was used to measure the maximal rate of change (slope) in absorbance at 405 nm over 20 minutes. A well containing protein C and thrombin only (i.e. no thrombomodulin) was used to determine the background APC concentration, and this was subtracted from all measurements. To quantify APC generated by thrombomodulin-modified SIS (SIS+TM), samples were compared to a standard curve of known APC concentrations.

2.5 In Vitro Stability

The stability of thrombomodulin activity on SIS+TM was assessed using *in vitro* methods. To determine the stability of TM activity under flow, a square of modified SIS (10 mm × 10 mm) was placed into a GlycoTech flow chamber with flow chamber dimensions of 0.254 mm height × 5 mm wide. A syringe pump was used to drive PBS over the SIS for 12 hours with a wall shear rate of 50 sec^{-1}. The APC generation of SIS+TM post-flow was compared to SIS+TM that had been kept in PBS under static conditions for the duration of flow. To determine if SIS+TM could be dried and maintain activity, the stability of thrombomodulin activity following a 24 hour drying was determined by comparing APC generation of the dried SIS to samples of freshly-modified SIS. The stability of SIS in plasma was determined by incubating 5 mm diameter discs of SIS+TM in baboon pooled plasma for times ranging from 5-90 minutes at 37°C. After incubation in plasma, discs of SIS were washed with PBS and the APC generation was compared to freshly-modified SIS.

2.6 Coagulation Assays

The coagulation of pooled, platelet-poor plasma was determined optically by measuring absorbance at 405 nm using a plate reader. Coagulation was stimulated using either tissue factor (Innovin®) or contact pathway activation (HemosIL®). Two different methods of initiating coagulation with these reagents were used. In the pro-coagulant solution assay, 5 mm diameter discs of modified SIS were placed into 96 well plates, followed by 50 μl of plasma and 100 μL of tissue factor or contact pathway activator diluted in 25 mM $CaCl_2$ (1:2000 for Innovin® and1:20 for the HemosIL®, respectively). Plates were immediately

transferred to a plate reader pre-warmed to 37 °C and coagulation was monitored optically by measuring the absorbance at 405 nm every 20 sec. The time at which the absorbance increased 20% from baseline value was considered to be the time of coagulation. Absorbance measurements were stopped at 45 min. Alternatively, a surface-driven coagulation assay was used to increase the sensitivity of the assays to surface-mediated anticoagulant activity. In this assay, initiation of coagulation was localized to the SIS surface by soaking discs of modified SIS for 1 hr with tissue factor or contact pathway activator diluted in TBS for 1 hr (1:100 for Innovin® and 1:1 for HemosIL®, respectively). The SIS discs were then washed 3 times with TBS, and 50 μl of plasma was added. Immediately after addition of the plasma, 100 μl of 25 mM $CaCl_2$ was added, and the plasma coagulation was monitored optically.

2.8 Baboon Ex Vivo *Arteriovenous Shunt*

Thrombus formation *in vivo* is governed by the transport of blood cells and proteins to and away from the developing thrombus.[30] To incorporate blood flow-dependent reactions into our hemocompatibility evaluation, we utilized a baboon femoral arteriovenous shunt that allows whole blood to flow across devices in the absence of anticoagulant or anti-platelet therapies.[31]–[33] Male baboons (*Papio anubis*) used in this study were cared for and housed at the Oregon National Primate Research Center at Oregon Health & Science University. Experiments were approved by the Oregon Health & Science University West Campus Institutional Animal Care and Use Committee according to the guidelines of the NIH “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (International Standard Book, Number 0-309-05377-3, 1996). A chronic extracorporeal shunt loop is implanted in the baboons between the femoral artery and the femoral vein. Prior to each study, autologous ^{111}In-labeled platelets and allogenic ^{125}I-labeled fibrinogen were infused into the baboon. Tubular SIS devices (4 mm dia, 5 cm long) were connected to the shunt loop and placed over a gamma camera (GE 400T gamma scintillation camera with Nuquest® software) for real-time measurement of platelet accumulation on the SIS devices. Blood flow is monitored using a transonic flow probe and kept constant at 100 ml/min using a regulator clamp downstream of the device. Following the 60 minute study, SIS devices were placed in 10% formalin and stored at 4°C until the ^{111}In had decayed >10 half-lives, at which point ^{125}I-labeled fibrinogen deposition was measured using a WIZARD automatic gamma counter (PerkinElmer). Platelet accumulation and fibrinogen deposition were measured on the central 2 cm of the graft to eliminate the potentially confounding effect of increased platelet accumulation at the connectors used to join the SIS device and shunt tubing.

2.9 Statistics

All data are reported as the mean ± standard deviation. Significant differences between groups were determined using a one-way ANOVA with Tukey's *post hoc* analysis. Letters are used in figures to indicate homogenous groups. In cases where only two sample groups were present, significant differences between groups was determined using a two-tailed student's t-test. Differences between groups were considered statistically significant if $p<0.05$.

3. Results

3.1 Thrombomodulin-modified SIS Generates APC

Modifying SIS with thrombomodulin (SIS+TM) catalyzed APC generation in a solution of protein C and thrombin (**Figure 2**). APC generation increased with greater concentrations of thrombomodulin, though no further increase in catalytic activity was seen at concentrations over 2 μg/ml. All future experiments with SIS+TM were done with a thrombomodulin concentration of 4 μg/ml. To compare the quantity of APC generated by SIS+TM to endothelial cells, baboon carotid ECs were cultured to confluence in a 96 well plate, and the APC generation assay was performed in parallel on these cells and with 5 mm diameter discs of SIS also in wells of a 96 well plate. Normalizing the APC generated to that of the endothelial cells, SIS+TM generated 5.46 ± 1.00-fold greater APC than the cells.

3.2 In Vitro *Thrombomodulin Stability*

The function of protein-based modifications, such as thrombomodulin, can be altered by exposure to mechanical forces and storage conditions. Therefore, the stability of thrombomodulin activity on SIS+TM was evaluated using *in vitro* testing systems. After thrombomodulin modification, SIS+TM was exposed to flowing PBS in a flow chamber for 12 hours at a shear rate of 50 sec^{-1}. Compared to samples kept under static conditions, APC generated by SIS+TM activity decreased from 260.2 ± 23.8 ng/cm^2 to 103.0 ± 79.0 ng/cm^2. Samples of SIS+TM that were left to dry for 24 hours retained TM activity, though APC generation decreased from 263.5 ± 40.2 ng/cm^2 to 131.1 ± 24.4 ng/cm^2. Based on these results, future studies with SIS+TM used freshly-modified SIS that was kept hydrated until the time of the experiment. After static incubation in blood plasma, SIS+TM decreased in activity by approximately 48%; however, no further loss in TM activity was observed over 90 minutes (Figure 3).

3.4 Plasma Coagulation

3.4.1 Thrombomodulin-modified SIS—The anticoagulant activity of biomaterials is generally determined by measuring the time it takes for blood plasma to coagulate following exposure to the biomaterial, and anticoagulant activity results in prolonged coagulation times. When plasma was stimulated to coagulate by a solution of either tissue factor or contact pathway activators, modifying SIS with thrombomodulin did not prolong coagulation, even at thrombomodulin concentrations considerably higher than the standard 4 μg/ml (Supplemental Figure S1). However, when surface-driven coagulation assays were used, which localized tissue factor or contact pathway activators to the SIS material, the thrombomodulin modification significantly prolonged coagulation at the standard concentration of 4 μg/ml (Figure 4).

3.4.1 Heparin-modified SIS—Modifying SIS with increasing concentrations of heparin resulted in a significant prolongation of blood plasma coagulation initiated by a solution of either tissue factor or contact activation. Heparin concentrations equal to and above 125 μg/mL prolonged plasma coagulation to times over 45 minutes (Figure 5). These *in vitro* results demonstrate that heparin-modified SIS (SIS+Hep) is a highly-effective anticoagulant modification compared to SIS+TM, which only had modest anticoagulant activity that was

limited to surface-initiated coagulation. All future experiments using SIS+Hep used 100 μg/mL heparin solution.

3.5 Dual-modified SIS Retains Full Thrombomodulin and Heparin Activities

SIS was modified with both thrombomodulin and heparin to enable both APC generation and heparin anticoagulant activity on SIS. To determine if the order of the modification affected the activities of thrombomodulin or heparin modifications, SIS was modified with either thrombomodulin or heparin first, followed sequentially with the second modification. SIS modified first with thrombomodulin then with heparin (SIS+TM+Hep) demonstrated APC generation similar to SIS+TM, and also prolonged tissue factor-activated plasma coagulation to a similar extent as SIS+Hep (Figure 6). Interestingly, SIS modified first with heparin and then with thrombomodulin (SIS+Hep+TM) demonstrated a reduction in both APC generation and heparin anticoagulant activity; thus, all future studies utilizing a dual modification performed the modifications sequentially with thrombomodulin first followed by heparin. The mechanical properties of the material were not noticeably altered by adsorption of either thrombomodulin, heparin, or the combination of the two in either order.

3.6 Dual-modified SIS Reduces Platelet Accumulation

Thrombus formation on biomaterials *in vivo* results from flow-mediated transport of cells and coagulation factors to and away from the biomaterial surface. To quantify the effects of the dual modification on thrombus formation, tubular devices constructed of unmodified SIS or SIS+TM+Hep were connected to a baboon arteriovenous shunt as described previously.[26] Platelets accumulated slowly on SIS devices over the course of the 1 hour study, with a total of $0.55 \pm 0.22 \times 10^9$ platelets·cm^{-1} at 60 minutes, and no devices occluded during the study (Figure 7). Devices constructed with the dual-modified SIS+TM +Hep had significantly less platelet accumulation over the 1 hour study, indicating the potent anti-thrombotic effect of the dual modification. Fibrin deposition was on average lower on SIS+TM+SIS devices than on SIS devices, but this trend was not significant (p=0.067).

4. Discussion

The decellularized biomaterial SIS has been investigated in a number of different vascular device applications. However, processes including thrombus formation and intimal hyperplasia have limited the function of some SIS-based devices. Vascular endothelial cells express multiple surface molecules to inhibit these processes, including the membrane protein thrombomodulin and the glycocalyx. This work applies thrombomodulin and the glycosaminoglycan heparin to SIS to enable APC generation, inhibit plasma coagulation, and reduce thrombus formation on SIS to advance the development of SIS-based vascular devices.

Modifying SIS with thrombomodulin enabled robust APC generation, and is consistent with prior studies that demonstrate adsorption is a feasible method of immobilizing thrombomodulin onto a material surface.[34] The amount of APC generated by a 5 mm disc of SIS+TM was approximately 5.5-fold greater than a monolayer of ECs cultured to confluence in a 6.4 mm diameter well (Figure 2). Due to the fibrous structure of SIS, the

total surface area of an SIS disc is difficult to determine. However, this result indicates that any application using SIS+TM biomaterial in a vascular device would generate a similar, if not greater, quantity of APC than an equivalent planar surface area of endothelium. From a biomaterials perspective, the absolute quantity of APC needed to have a physiologic effect likely depends on many factors specific to the application (blood flow rates, presence of systemic anticoagulation, local endothelial injury, etc.) and therefore would require application-specific *in vivo* testing. These results that show SIS+TM has comparable APC generation to carotid ECs are encouraging and support further studies to determine the modification's efficacy in specific vascular applications.

Two coagulation assays used clinically to monitor anticoagulant dosing or to identify coagulation factor deficiencies are the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which identify inhibition or deficiency of the tissue factor and contact pathways, respectively. The PT and APTT reagents utilize high concentrations of procoagulant stimuli to enable rapid coagulation times for clinical diagnostics; however, these assays are not sensitive to subtle anticoagulant activity. Due to their lack of sensitivity to material properties, these assays are often not recommended to demonstrate hemocompatibility of biomaterials.[35] Here, the PT and APTT reagents were diluted and coagulation was measured optically to permit longer coagulation times (on the order of minutes) that are closer to physiological clotting times. Initial optical coagulation measurements that added pro-coagulant solutions of tissue factor or contact pathway activator to the bulk plasma solution did not reflect any anticoagulant activity of the SIS +TM compared to unmodified SIS. However, thrombomodulin is stable on SIS in plasma (Figure 3) and the anticoagulant activity is catalyzed at the surface of the material. Because thrombus formation *in vivo* initiates at the material or vessel surface rather than in the bulk blood flow, the assay was refined to localize the pro-coagulant stimuli to the material surface. Using this surface-driven coagulation assay, SIS+TM significantly prolonged coagulation initiated by tissue factor or contact pathway activation compared to unmodified SIS (Figure 4). Importantly, this prolongation was due to TM activity rather than reduced pro-coagulant adsorption due to previous TM adsorption on SIS (Supplemental Figure S2). The discrepancy in these assays highlights the importance of designing coagulation experiments that appropriately test for surface-initiated anti-thrombotic activity, such as catalyzed by SIS+TM, versus systemic anticoagulation therapy that affects the entire plasma volume.

Heparin exerts anticoagulant activity by binding to ATIII and markedly accelerating the ATIII-dependent inhibition of FXIIa, FXIa, FXa, FIXa and thrombin. This work determined that modifying SIS with exogenous heparin confers potent anticoagulant activity to SIS (Figure 5). Although SIS contains some endogenous heparin,[26][36] the addition of exogenous heparin significantly enhanced the anticoagulant activity compared to the unmodified material. A dual modification of SIS with both thrombomodulin and heparin, performed sequentially in that order, confers both robust APC generation as well as anticoagulant activity on SIS. The anticoagulant activity of SIS+Hep was more potent than the SIS+TM in all *in vitro* assays that measured platelet poor plasma coagulation times. However, considering the wealth of literature describing the protective effects of APC on the vascular endothelium,[17][37][38] a biomaterial modification that enables APC generation

may confer protective effects on endothelial cells and is therefore only evident following implantation into blood vessels. Other applications using thrombomodulin-modified materials have shown reduced thrombosis and intimal hyperplasia surrounding the implant,[39] and an *in vivo* model could demonstrate if these findings translate to SIS-based devices.

A baboon arteriovenous shunt was used to measure thrombus formation on SIS using flowing whole blood. Due to the limited number of available baboons, not all of the individual modifications were able to be tested. As such, only unmodified SIS and the dual modification SIS+TM+Hep, which facilitated robust APC generation as well as heparin-mediated anticoagulation, were assessed in this experiment (Figure 6). The physical shunt configuration and lack of anticoagulant or anti-platelet drugs results in rapid platelet deposition such that even clinically-approved materials and devices have substantial platelet accumulation over a 60 minute study.[31][40] Consistent with previous results, overall thrombus formation on SIS was low, with a total of $0.55 \pm 0.22 \times 10^9$ platelets·cm^{-1} on SIS devices at 60 minutes (Figure 7). This quantity of platelets is similar to the amount of platelets that accumulated on the common clinically-utilized vascular graft material expanded polytetrafluoroethylene (ePTFE) in prior studies by our group using this model.[41] Despite the low platelet accumulation of unmodified SIS, the dual-modified SIS +TM+Hep did demonstrate a significant reduction in platelet accumulation, highlighting the potent anti-thrombotic effect of the dual modification.

One interesting result from this study was that the order of the heparin and thrombomodulin modifications had a significant effect on both APC generation and anticoagulant activity (Figure 6). This was particularly surprising since heparin has been used as a linker molecule to increase the binding of growth factors onto biomaterials.[42][43] Although the exact mechanism responsible for the ordering effect was not determined in this study, the reduced APC generation of SIS first modified with heparin may be due to repulsion of the negatively-charged heparin and the chondroitin sulfate glycosaminoglycan on the thrombomodulin protein resulting in reduced thrombomodulin adsorption. If that is the case, repulsion of heparin by previously-adsorbed thrombomodulin, as would theoretically occur with SIS+TM+Hep, may be present but limited due to the considerably lower concentration of thrombomodulin used (4 μg/ml thrombomodulin vs 100 μg/ml heparin). Alternatively, rather than affecting the adsorbed quantity of either molecule, the order of adsorption may affect the availability of the binding sites of the molecules. For example, reduced APC generation of SIS+Hep+TM compared to SIS+TM+Hep may be due to heparin inhibiting thrombomodulin binding of thrombin. Both of these explanations are theoretical, and additional work is needed to clarify the mechanism by which the order of adsorption has such a pronounced effect on the bioactivity of this modification.

In conclusion, modifying SIS with TM enabled APC generation to a degree that is comparable to an equivalent area of arterial vascular endothelial cells. SIS+Hep resulted in potent anticoagulant activity that significantly prolonged coagulation initiated by either tissue factor or contact pathway activation. SIS dual-modified sequentially with TM and then heparin maintained robust APC generation and anticoagulant activity. The effect of this dual-modification in reducing thrombus formation was demonstrated by the significant

reduction in platelet accumulation on SIS+TM+Hep compared to unmodified SIS in a baboon *ex vivo* arteriovenous shunt model. This body of work demonstrates a dual-acting modification of SIS that, by enabling APC generation and inhibiting thrombus formation, may be useful in a variety of vascular devices. Future work may investigate SIS+TM+Hep *in vivo* to determine the effects of the dual modification on long-term biomaterial remodeling and ability to preserve vascular endothelial cell integrity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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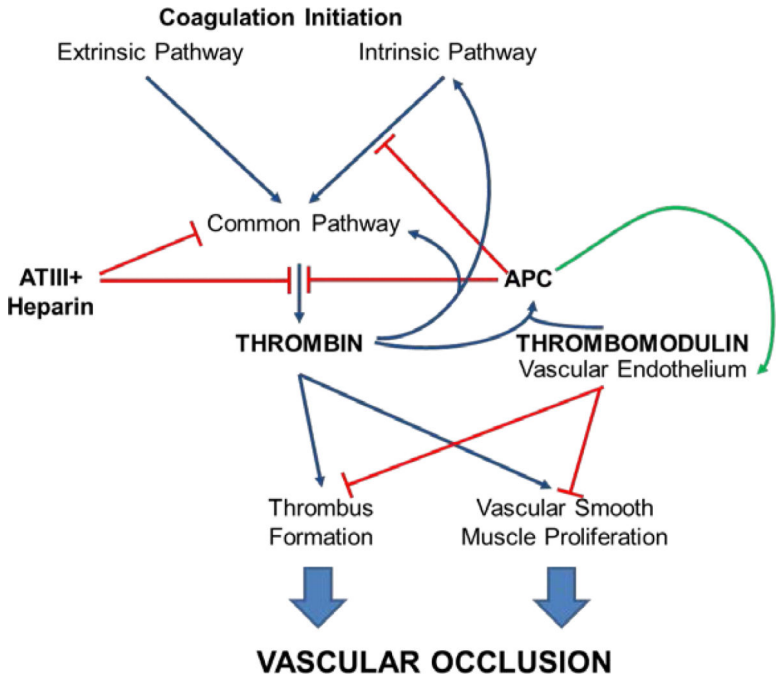

Figure 1. Role of heparin, thrombomodulin and APC thrombosis

Blue arrows represent an activating role, red arrows represent an inhibitory role, and the green arrow represents a protective role, specifically against inflammation-induced apoptosis. Heparin binds antithrombin III (ATIII) to accelerate inhibition of coagulation factors in the intrinsic and common pathways. APC inhibits processes contributing to thrombosis by inhibiting thrombin activation and the downstream effects, as well as by protecting proper vascular endothelial function. Thrombomodulin, expressed on the vascular endothelium, is the major catalyst for APC generation.

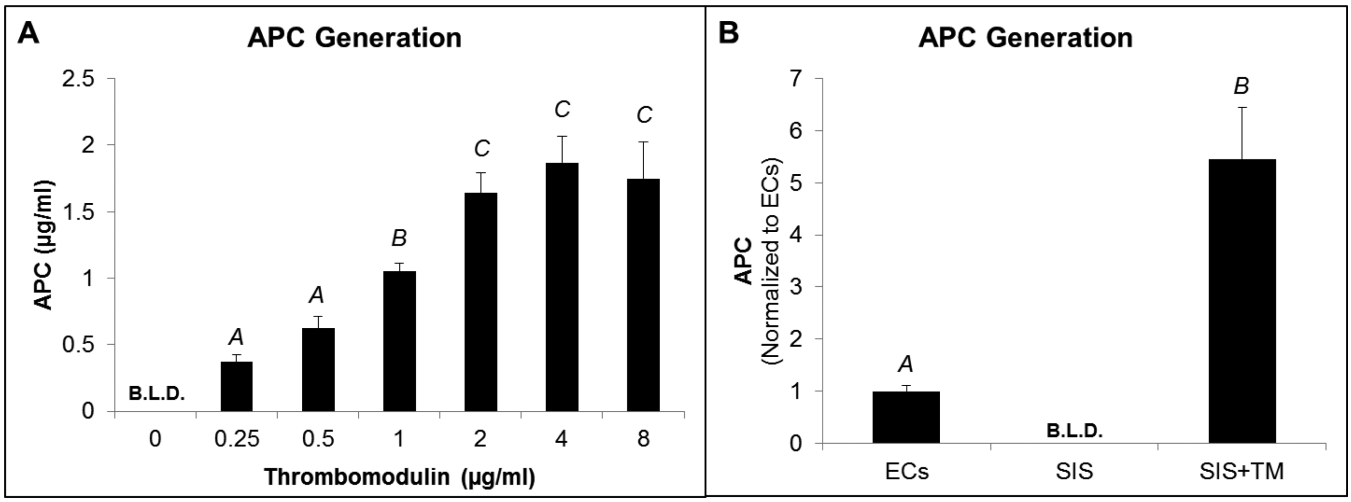

Figure 2. APC Generation of SIS+TM

Samples were incubated in a solution of protein C and thrombin for 1 hour, the reaction was quenched with hirudin, and the amount of APC generated was quantified using the chromogenic substrate S-2366. (A) The quantity of APC generated by SIS+TM increased with increasing concentrations of thrombomodulin solution up to 2 µg/mL. (B) The APC generated by a 5 mm disc of SIS in a 96-well plate was approximately 5.5-fold greater than a confluent layer of ECs in a 96 well plate. “B.L.D.”, below limit of detection. Letters indicate homogenous subsets based on a one-way ANOVA and Tukey's *post hoc,* n=4 (A) and n=3 (B).

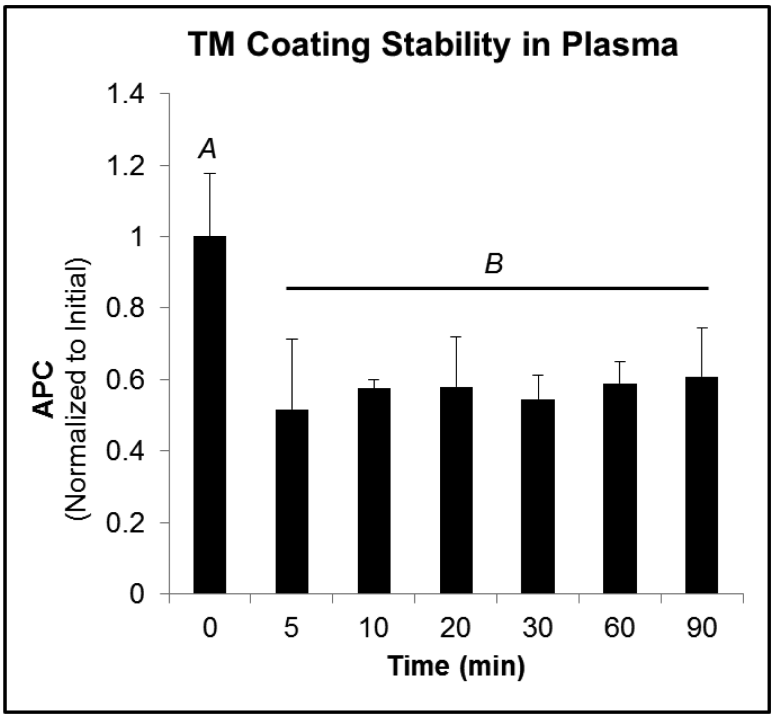

Figure 3. Activity of SIS+TM following incubation in plasma

The SIS+TM biomaterial was incubated in plasma at 37°C for the specified time, and APC generation of the modified SIS was quantified post-incubation. After 5 minutes, a reduction in TM-catalyzed APC generation of approximately 48% was observed; however, no further loss in TM activity occurred over the 90 minute treatment. Letters indicate homogenous subsets based on a one-way ANOVA and Tukey's *post hoc,* n=3.

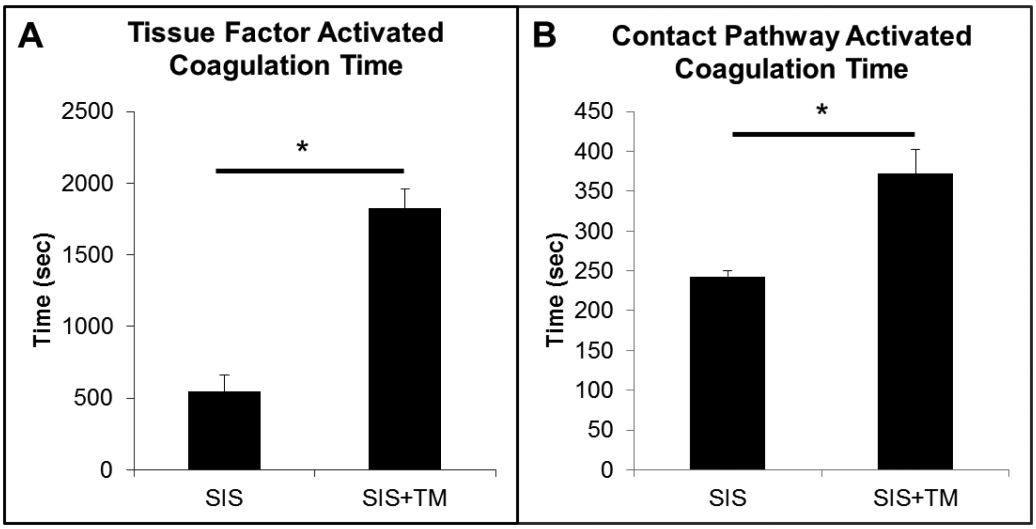

Figure 4. Plasma coagulation times of surface-driven coagulation reactions

SIS and SIS+TM were soaked in (A) tissue factor or (B) contact activation solutions to localize pro coagulant stimuli to the material surface. Platelet-poor plasma was then added to samples, and the time to coagulation was determined optically using a plate reader. Compared to unmodified SIS, SIS+TM prolonged plasma coagulation promoted by either tissue factor or contact pathway. "*" indicates significantly different groups, $p<0.05$ by two-tailed student's t-test, n=4.

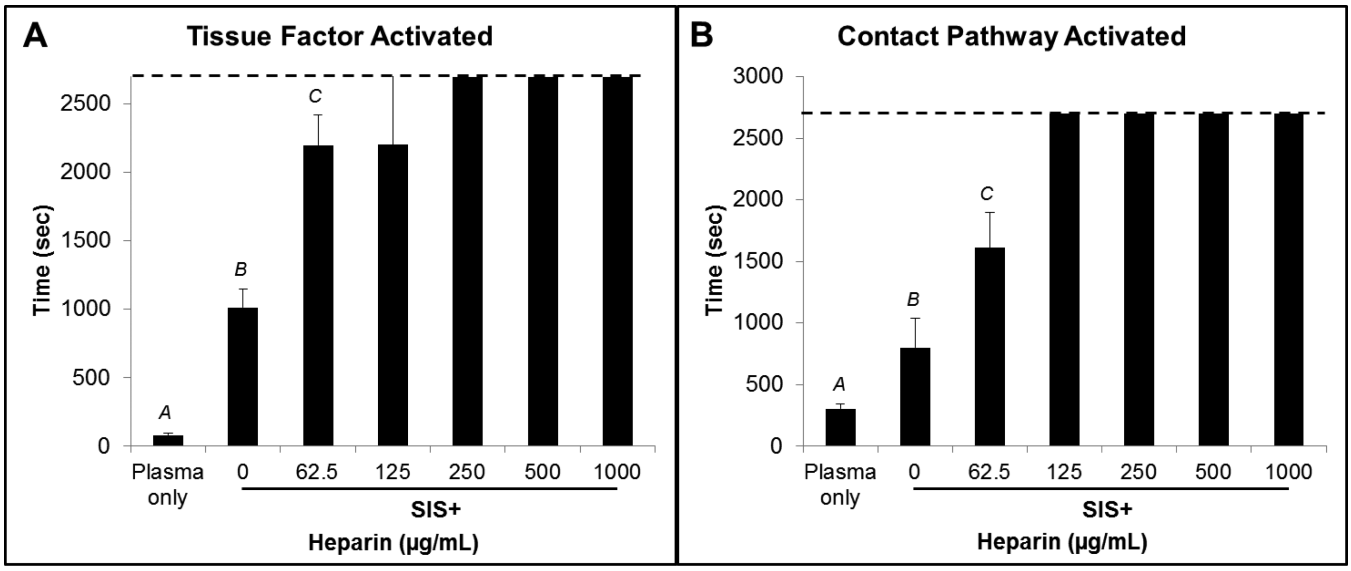

Figure 5. Plasma coagulation times of pro-coagulant solution driven coagulation when incubated with SIS+Hep

Samples were placed into platelet poor plasma, and pro-coagulant solutions of tissue factor (A) or contact pathway activator (B) were used to stimulate coagulation. Increasing heparin concentrations led to more prolonged blood plasma coagulation initiated by either pathway. Letters indicate homogenous subsets based on a one way ANOVA and Tukey's *post hoc,* n=4.

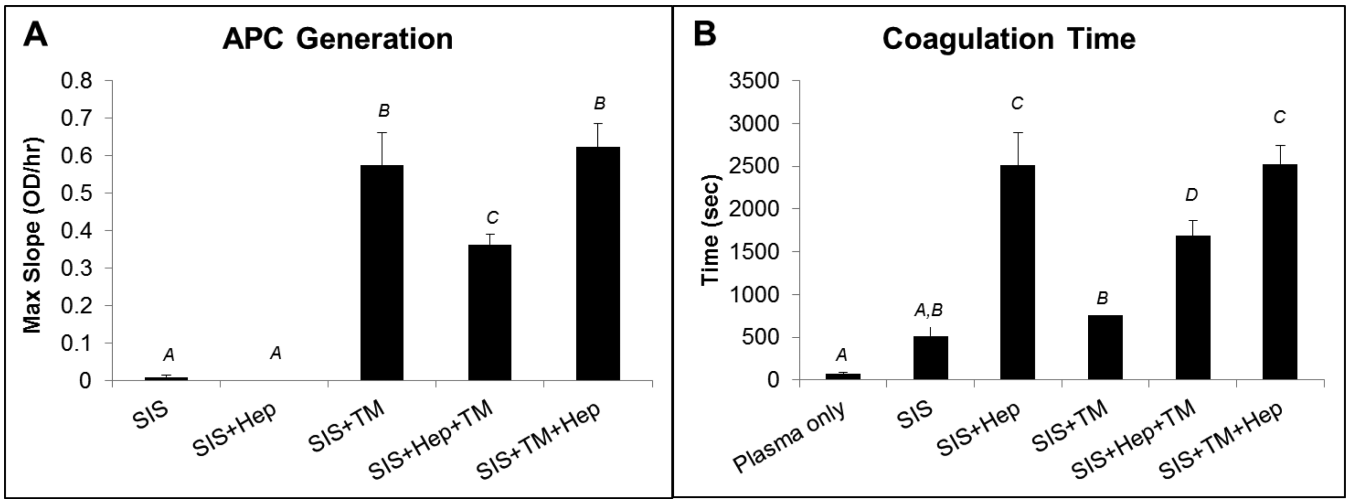

Figure 6. APC generation and plasma coagulation times of pro-coagulant solution driven coagulation when incubated with dual-modified SIS

SIS modified with thrombomodulin then heparin (SIS+TM+Hep) demonstrated APC generation similarly to SIS+TM, and also prolonged pro-coagulant solution driven plasma coagulation to a similar extent as SIS+Hep. Conversely, SIS modified heparin followed by thrombomodulin (SIS+Hep+TM) had lower APC generation and anticoagulant activity. Letters indicate homogenous subsets, $p<0.05$ via one-way ANOVA and Tukey's post-hoc, n =4.

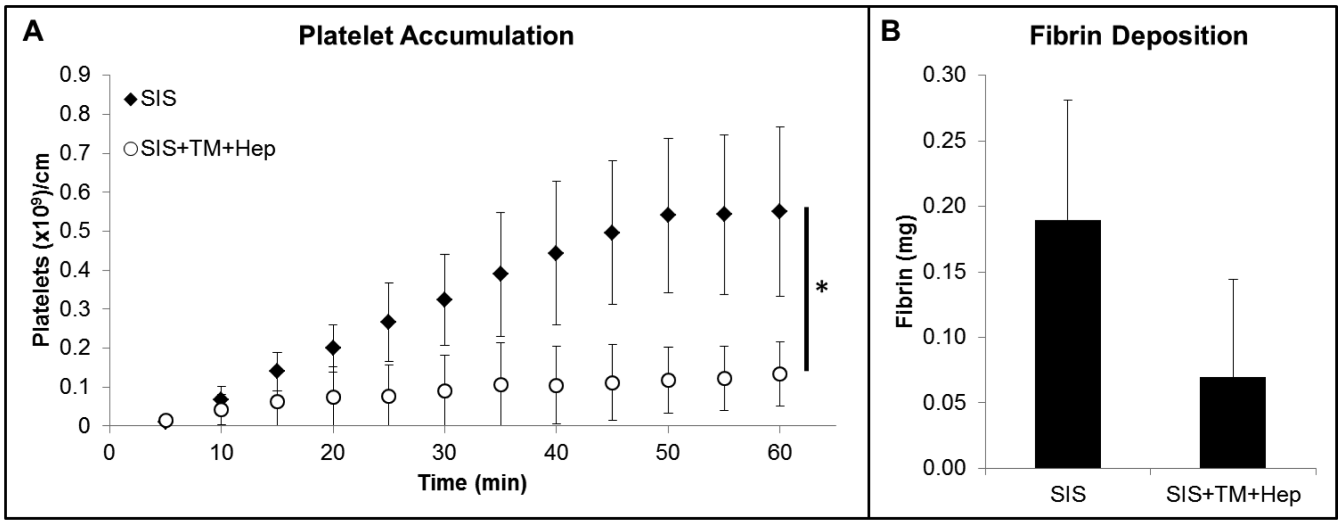

Figure 7. Platelet accumulation and fibrin deposition on SIS and SIS+TM+Hep devices

Tubular SIS devices were connected to a baboon arteriovenous shunt. The accumulation of ^{111}In-labeled platelets was measure in real time, and the total amount of ^{125}I-labeld fibrin was measured following the study. SIS+TM+Hep had significantly less platelet accumulation after 60 minute exposure to blood. Fibrin deposition was not significantly different between SIS and SIS+TM+Hep (p=0.067). "*" indicates significantly different platelet accumulation based on a two-tailed student's t-test not assuming equal variance, $p<0.05$, n=4.