Glycocalyx-Like Hydrogel Coatings for Small Diameter Vascular Grafts

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Novel biological vascular conduits, such as decellularized tissue engineered vascular grafts (TEVGs) are hindered by high thrombogenicity. To mimic the antithrombogenic surface of native vessels with a continuous glycosaminoglycan layer that is present on endothelial cells (ECs), a hyaluronic acid (HA) modified surface is established, to effectively shield blood platelets from collagen-triggered activation. Using the amine groups present on 4 mm diameter decellularized TEVGs, a continuous HA hydrogel coating is built via a bifunctional thiol-reactive cross-linker, thereby avoiding nonspecific collagen matrix cross-linking. The HA hydrogel layer recreates a luminal wall, “hiding” exposed collagen from the bloodstream. In vitro blood tests show that adhered platelets, fibrinogen absorption, and fibrin formation on HA-coated decellularized TEVGs are significantly lower than on uncoated decellularized TEVGs. The HA surface also inhibits macrophage adhesion in vitro. HA-coated decellularized syngeneic rat aortae (≈1.5 mm diameter), and TEVGs in rat and canine models, respectively, are protected from aggressive thrombus formation, and preserve normal blood flow. Re-endothelialization is also observed. HA-coated TEVGs may be an off-the-shelf small-diameter vascular graft with dual benefits: antithrombogenic protection and promotion of endothelium.

1. Introduction

Development of a small-diameter tissue engineered vascular graft (TEVG) is an unmet medical need that has been under development since before the 1980s.[1–3] The objective of designing a nonthrombogenic small diameter vascular graft is centered on modifications of the inner surface of the vascular prosthesis to prevent thrombogenicity, through physical and/or chemical methods.[4–7] Of the various surface modifications developed to reduce the thrombogenicity, such as heparin bonding,[8] endothelial cell (EC) seeding,[9] and carbon co-extrusion,[10] have demonstrated the most impactful clinical results.[11] However, even these modifications have shown disappointing improvements in patient outcomes and life prolongation. For instance, 6 mm diameter carbon-coated ePTFE grafts (Carboflo) only marginally increased ePTFE primary patency from 30% to 33%.[12] The more biological approach of seeding patient-sourced ECs onto ePTFE implants failed to demonstrate significant improvement in over 400 human subjects, and thrombosis remained the primary mode of failure.[13] A functional success has been observed with covalent heparin bonding to ePTFE (Propaten, Gore Inc.). However, this patency improvement remains marginal, with less than 7% increase in primary patency of Propaten grafts as compared to standard ePTFE.[14]
These observations motivated the development of a novel antithrombogenic coating method, with a goal of producing better-functioning vascular grafts.[13,15,16] As vascular grafting approaches are expanding to include tissue-engineered conduits that are in clinical development,[16,17] it seems appropriate to evaluate the impact of novel coatings for engineered vascular tissues. For instance, thrombomodulin and tissue factor pathway inhibitors that are bound to decellularized TEVG have shown reduction of platelet adherence and activation in vitro, but no preclinical or animal work has been described.[13,18]

Of the tissue engineered conduits currently in clinical development, the most widely clinically studied comprise decellularized, engineered biological grafts.[19] Our TEVG is grown in a bioreactor using human smooth muscle cells (SMCs) that are seeded onto a degradable polymer and grown under pulsatile conditions that simulate blood flow. The cultured TEVG is then decellularized, thereby producing an arterial graft which lacks cellular antigens, but retains surgically compatible mechanical properties.[13,20] Using this technology, over 200 arteriovenous grafts have been implanted in hemodialysis patients in ongoing clinical trials. Reported primary patency at 1 year in 60 patients was 28%, with secondary patency of 89%.[19] Therefore, TEVGs in the dialysis setting suffer from acute thrombosis, although secondary patency is substantially better.

Acute thrombosis remains the fundamental weakness for all available graft materials, both synthetic[17] and tissue engineered.[19] In the arterial circulation, thrombosis is primarily a platelet-driven event, as platelets tether to collagen surfaces by binding to specific collagen receptors, including Ib-IX-V which binds von Willebrand factor (vWF).[21] Given that the surface of decellularized TEVGs is primarily collagen, platelet binding and activation is likely a key driver of observed graft thromboses. After binding and activation, amplification of platelet response and platelet aggregation is supported by factors such as adenosine 5’-diphosphate (ADP), thrombin, and thromboxane A2.[22] In healthy native vessels, platelet adhesion to collagen is prevented by both the endothelium and by the luminal glycocalyx layer. However, prior to in vivo re-endothelialization, decellularized-engineered grafts are vulnerable to platelet activation due to exposed collagen in the wall of the graft. Therefore, the primary failure mode of decellularized TEVGs is thrombosis, which may restrict their usage to larger diameter applications (≥6 mm).

Prior reports describe hyaluronic acid (HA) coated onto non-bioactive polymers can inhibit platelet adhesion and bacterial activity, and have been shown to promote EC attachment.[23–25] However, such coatings have not been extensively studied for antithrombosis or using vivo studies. Hyaluronic acid coated on a stainless-steel stent in a baboon arteriovenous shunt performed effective antithrombosis with only 2 h, as assessed by scanning electron microscope (SEM).[26]

Our goal for supplying a degradable coating based upon HA was to provide a surface that blocked platelet activation in the short and intermediate term, while providing a means for ECs to eventually adhere to the fibrous collagen substrate of the vessel lumen over time. It may enable an “off the-shelf” TEVG for small diameter vascular applications. In this work, we have characterized the coating architecture, surface chemistry, stability, mechanical properties, and antithrombogenicity. Furthermore, we assessed the functionality of luminal HA-coating on decellularized native allogeneic arteries and TEVGs, and surgically implanted these grafts into both rodent and canine vascular models.

2. Results

2.1. Creating a Structurally Uniform HA Coating

Coating design, described in Figure 1, was engineered to take advantage of HA’s antithrombogenicity and pro-endothelialization properties without inducing nonspecific cross-linking within the collagenous graft (either decellularized TEVGs or decellularized native rat aorta). Figure S1, Supporting Information, shows that no nuclei are present in the decellularized TEVGs (hematoxylin & eosin, H&E stain) and the structure of the decellularized TEVGs consists primarily of collagen (Trichrome stain). Briefly, succinimidyl-([N-maleimidopropionanimo]-diethyleneglycol] ester ( NHS-PEG-maleimide, Thermo scientific) (Figure 1a, 2) was used as a transition layer, and was coated on the decellularized TEVG or rat aorta for 30 min (Figure 1a, 1), thereby modifying the exposed primary amines with maleimide groups, which are thiol-reactive (Figure 1a, 3). Covalent attachment of thiol-functionalized hyaluronan (Figure 1b, 4) onto the thiol reactive graft/vessel (Figure 1b, 3) was accomplished by incubating the grafts in the presence of thiol-modified sodium hyaluronate (HA Glycosil, ESI-BIO) (Figure 1b, 5). The thiolated HA was continuously perfused over the graft/vessel for 1 h, allowing disulfide bridge formation in the HA to form a thin and uniform layer on the lumen of the vessel/graft (Figure 1c, 6). All solutions were perfused via a custom-built perfusion bioreactor, where the decellularized grafts were mounted end-to-end, and solutions were injected into a closed loop system that was continuously perfused via a peristaltic pump (Figure S2, Supporting Information).

Decellularized rat aorta and TEVG surface characterization was performed using amine-reactive iron nanoparticles (Figure S3, Supporting Information). Iron nanoparticle adhesion to surfaces demonstrated the presence of only sparse amine groups, with approximately one amine group per square micrometer. This observation helped to define the coating design, including the necessity of an anchoring cross-linker for the large HA molecules. Reactivating the amines into thiol-reactive molecules (maleimides) with a bifunctional cross-linker allowed the subsequent covalent binding of the thiolated HA building the coating’s first layer. Following attachment of the maleimide moieties, thiol groups on HA react with the maleimides. Unreacted thiol groups go onto form disulfide bonds between the HA chains. The cross-linking of HA to HA molecules builds a 5 µm thick continuous viscoelastic HA hydrogel layer on the graft surface. Coated and uncoated TEVGs were analyzed by transmission electron microscopy (TEM), scanning electron microscopy (SEM), and toluidine blue histology staining. The topography and overall morphology showed a substantial difference imparted by the HA coating (Figure 2a–e). As expected, decellularization resulted in exposure of collagen fibers on the lumen (Figure 3a). Conversely, the HA-coated
lumen is protected by a continuous, smooth HA-based fibrous structure, which “hides” the otherwise exposed collagen fibers (Figure 3b). Toluidine blue stains revealed the continuity of the dense coating with a uniform thickness of \(5 \mu \text{m}\) (Figure 3d). Figure S4, Supporting Information, shows the continuity and uniformity of the coating of a full-view cross-section. SEM “en face” view validated the HA hydrogel coating appearance as smooth (Figure 3f), in contrast to collagen fibers (Figure 3e).

HA coating made the surface of the TEVG more hydrophilic, with a decrease in the water contact angle from \(103.1 \pm 1.4^\circ\) to \(12.8 \pm 2.2^\circ\) (Figure 2g,h). Correspondingly, there was an increase in the polar surface energy from \(0.2 \pm 0.1\) to \(50.0 \pm 0.9 \text{ mJ m}^{-2}\).
Figure 2. Coating morphology and properties. Transmission electron microscopy (TEM) images of the lumen of a) uncoated and b) hyaluronic acid (HA) coated tissue engineered vascular graft (TEVG). Toluidine blue stain of c) uncoated and d) HA-coated TEVGs. Scanning electron microscope (SEM) “en face” views of TEVG prior to coating, showing e) the exposed collagen fibers and f) the gel-like lumen morphology post coating. Scale bars represent 10 µm. Water contact angles of g) uncoated and h) HA-coated TEVGs. i) Surface energy of uncoated and HA-coated TEVGs, indicating a high hydration surface of HA coating. j) Tensile mechanical properties, uncoated (black line), thiol-reactive (green line), and hyaluronan-functionalized TEVGs (blue line), showing no significant effect of coating process on the ultimate strengths and Young’s moduli. k) Coating stability in cell culture medium (M199) with 50 U mL⁻¹ hyaluronidases for 2 weeks at 37 °C. l) Coating stability in fresh blood plasma for 2 weeks at 37 °C. m) Coating stability in PBS at 4 °C for 1 year storage. The mean ± s.d. is shown, N = 3 graphs (g–i).
Figure 3. In vitro hemocompatibility and cytocompatibility tests. Scanning electron microscope (SEM) and rhodamine images of human platelet adhesion to a,b,e,f) uncoated and c,d,g,h) hyaluronic acid (HA) coated tissue engineered vascular grafts (TEVGs) for 30 min. i) Quantitative analysis of average number of adhered platelets \((N = 3, **p < 0.01\) by unpaired, two-sided t-test with Welch's correction). SEM images of the j,k) uncoated and l,m) HA-coated TEVGs exposed to whole human blood for 30 min. Fibrinogen staining of n,o) uncoated and p,q) HA coated TEVGs after 1 h of incubation with \(50 \mu g\) mL\(^{-1}\) fibrinogen in PBS. \((N = 3, *p < 0.05\) by unpaired, two-sided t-test with Welch's correction). SEM images of fibrin formation of s,t) uncoated and u,v) HA-coated TEVGs after 15-min incubation with the whole blood with premixed \(8 \times 10^{-3}\) m \(CaCl_2\) and 0.25 U mL\(^{-1}\) thrombin. w) Quantification of adhered fibrin \((N = 3, ****p < 0.0001\) by unpaired, two-sided t-test with Welch's correction). x) The concentrations of the pro-thrombin F1+2 fragment \((N = 3, ns, by unpaired, two-sided t-test with Welch's correction) after 3-h blood contact. Less adhered platelets, fibrinogen absorption, and fibrin formation on HA-coated TEVGs were significantly lower than on uncoated TEVGs. CD68 immunostaining of y, aa) uncoated and z, bb) HA-coated TEVGs exposed to murine macrophages in the medium with 90 U mL\(^{-1}\) hyaluronidase for 2 and 24 h; quantitative analysis cc) of the average number of adhered macrophages, showing the much less adhered macrophages on the HA-coated graft than that on the uncoated graft. \((N = 3, *p < 0.05\) by two-way ANOVA with Bonferroni correction). All the statistical analysis in this figure, the mean ± s.d. is shown.
(Figure 2i). The high hydration of HA, due to the presence of large numbers of hydrophilic groups in the polymer chain, including hydroxyl and carboxyl groups, likely underlies the increased hydrophilicity of treated surfaces.

To determine whether the cross-linking reaction in the formation of the HA hydrogel impacted the bulk mechanics of the decellularized graft, it was necessary to perform stress–strain tensile testing of the uncoated (1 in Figures 1a and 2j black line), thiol-reactive (3 in Figures 1a,b and 2j green line), and hyaluronan-functionalized TEVGs (6 in Figures 1c and 2j blue line). Mechanical testing revealed that there was minor change in ultimate strengths among the uncoated controls (2.33 ± 0.41 MPa), thiol-reactive grafts (2.71 ± 0.20 MPa), and hyaluronan-functionalized grafts (2.88 ± 0.43 MPa). There is no statistically significantly difference (N = 3, p > 0.05). The Young’s moduli among the uncoated controls (4.14 ± 0.43 MPa), thiol-reactive grafts (3.80 ± 1.88 MPa), and hyaluronan-functionalized grafts (3.96 ± 1.40 MPa) were also not statistically significantly different (N = 3, p > 0.05). These data support the hypothesis that there is a preservation of graft wall mechanical properties after coating (i.e., there is a lack of nonspecific graft wall collagen cross-linking).

To quantify the mass of the HA coating, the HA-coated TEVGs were enzymatically digested using collagenase and hyaluronidase, and the total amounts of covalently bound HA and thiol groups were quantified via liquid chromatography–mass spectrometry (LC–MS) assay. However, the LC–MS estimated the total amount of HA as 200 µg cm⁻².

To test the stability of the HA coating, the HA-coated TEVGs were assessed under three separate conditions: storage in cell culture medium with 90 U mL⁻¹ hyaluronidases; fresh blood plasma (containing naturally occurring hyaluronidases); and PBS. After incubation in one of the three types of incubating media, coating thickness was quantified by staining with toluidine blue. A total of 36.5 ± 8.0% of the coating thickness remained on the vascular grafts after 2 weeks when exposed to cell culture medium with 90 U mL⁻¹ hyaluronidases at 37 °C (Figure 2k, N = 3). Assessing the coating stability under fresh blood plasma indicated that, even after 2 weeks of fresh plasma incubation with daily plasma changes, 53.0 ± 9.9% of the coating thickness remained on the vascular grafts (Figure 2l, N = 3). This supported the potential coating protection of the decellularized surface after prolonged blood exposure.[27] Assessing the coating stability under storage at 4 °C in PBS showed that 1 year of storage had a negligible impact on coating thickness (Figure 2m). This shows that the coating may be very stable and useful in the setting of prolonged storage of coated surfaces for eventual clinical application.[28]

2.2. In Vitro Assessment of the HA Coating

Using human isolated platelets and whole blood, the HA coating’s capacity to prevent in vitro thrombus formation was characterized. After 30 min of exposure to human platelets, representative SEM images show a large number of activated platelets with the characteristic extending filopodia in the absence of HA coating (Figure 3a,b). By contrast, HA coating significantly prevented platelet adhesion, and the few adhered platelets on the HA-coated surface displayed a round, isolated, and nonactivated morphology (Figure 3c,d). Rhodamine staining of adhered platelets was consistent with SEM results (Figure 3e–h). Quantification based on the rhodamine staining showed ~4000 platelets cm⁻² on the uncoated graft and ~50 platelets cm⁻² on the HA-coated graft (Figure 3i, N = 3, p < 0.001). After 30 min of exposure to whole blood, erythrocytes and platelets aggregated on the uncoated graft, forming fibrous-like thrombus structures (Figure 3j,k), in contrast to the HA-coated graft (Figure 3l,m). After 1 h of exposure to fibrinogen from human plasma, representative fluorescence images show a large amount of fibrinogen adsorbed onto the TEVG surface (Figure 3n,o), while almost nothing adsorbed on the HA-coated surface (Figure 3p,q). Fluorescence intensity of the fibrinogen adsorption on the TEVGs was more than on HA-coated grafts (Figure 3r, N = 3, p < 0.01). After 2 h of exposure to whole blood that was premixed with 8 × 10⁻³ m CaCl₂ and 0.25 U mL⁻¹ thrombin, representative SEM images show a large amount of polymerized fibrin and aggregated red cells on the TEVG surface (Figure 3s,t). In contrast, only a few fibrin strands can be found on the HA-treated surface (Figure 3u,v). The fibrin density on the TEVGs was two orders of magnitude higher than that on the HA coating surface per square millimeter (Figure 3w, N = 3, p < 0.0001). Furthermore, very low concentrations of the prothrombin F1+2 fragment (Figure 3x, N = 3, ns) after the 3 h blood with 3.2% sodium citrate incubation indicate that the thrombin generation loop suppresses both HA and TEVG surfaces,[29] probably due to the citrate as a strong anticoagulant in the incubating blood blocks the coagulation process too much. Overall, these blood tests suggested that the HA-coated graft lumen can be nonthrombogenic and inhibit blood clotting within the graft.

Inflammation, especially stimulated by macrophages, plays a critical role in microvessel formation and wound healing, and may impact remodeling and stenosis in TEVGs.[30] Therefore, to assess whether the HA coating may stimulate macrophage adhesion, the effect of HA degradation on macrophage adhesion was evaluated. The culture medium used for this study contained 90 U mL⁻¹ hyaluronidase to accelerate HA degradation. As presented in Figure 2k, the thickness of HA coating decreased from 5.45 ± 1.06 µm to 5.18 ± 0.26 µm in 24 h under hyaluronidase exposure, thereby demonstrating some breakdown of the polymerized HA. In this setting, the average number of adhered macrophages on the uncoated TEVGs was significantly more than that on the HA-coated TEVGs at both 2 h (p < 0.01) and 24 h (p < 0.05) (Figure 3y–cc), implying that a degrading HA coating may not be a strong stimulus for macrophage adhesion and accumulation on the graft lumen.

2.3. HA Coating Reduces Thrombogenicity in a Rat Model

HA-coated and uncoated decellularized rat allograft aortas were implanted in the rat infra-renal aorta (Figure 4a, n = 12 total, 6 in each group) without any systemic anticoagulation for 1 month. Obvious differences between the two groups indicated a functional effect of the coating. Control, uncoated grafts (5 of 6) suffered from thrombosis as evidenced by either a complete loss of blood flow, or dramatically reduced (<3 cm s⁻¹...
peak velocity) blood flow velocities observed by Doppler ultrasound (Figure 4b,c). Some rats developed collateral circulation as a response to the absence of flow in the abdominal aortae. In contrast, 6 of 6 cases of HA-coated grafts remained patent, with relatively unchanged peak flow velocities of approximately 18 cm s⁻¹ (Figure 4b,c).

Explanted mid-graft and anastomotic sections were analyzed by digital morphometry, to determine the wall area, lumen
area, wall thickness, neointimal pannus area, and the diameter of the implant lumen for each sample. The reduced blood flow (functionally measured by Doppler) in control, uncoated grafts was corroborated by the quantitative image analysis of the explants. Quantitative H&E image analysis indicated a four-fold or greater percentage of patent lumen area of the coated grafts as compared to uncoated (Figure 4d, \( n = 6, *p < 0.05 \)), while uncoated grafts were dilated relative to coated grafts (Figure 4f, \( n = 6, **p < 0.01 \)).

Histological observations further supported the functional findings. An intact and continuous HA coating on the coated TEVG explant was observed through the entire vessel by toluidine blue staining (Figure 4b). The coating thickness kept around 2.0 ± 1.4 \( \mu \)m, showing that the coating thickness remained during 1 month rat implantation. Uncoated control grafts accumulated significant intimal tissue (Figure 4i), with circumferentially organized layers of alpha-smooth muscle actin (\( \alpha \)-SMA) positive cells comprising the neointima (Figure 4m).

Conversely, a lack of neointimal formation (Figure 4i), in the setting of EC repopulation, was observed in the partial lumens of coated grafts (Figure 4n). The coating therefore appeared to prevent thrombus formation, perhaps while limiting the ingrowth of \( \alpha \)-SMA positive cells. The HA-coated grafts were either void of cells on the lumen (HA coating persistent on 3 of 6 cases for 1 month) or were lined with cells expressing the EC marker vWF. CD68 and CD45 costaining indicated that the coating also diminished the leukocyte accumulation at 30 days in the rat experiments (Figure 4o,p).

In terms of the architecture of the vascular wall, the control graft wall elastin fibers were somewhat less organized in the uncoated as compared to coated grafts, as seen by EVG staining (Figure 4k,l).

### 2.4. HA Coating Reduces Thrombogenicity in Canine Model

As the rodent model is not highly predictive of graft function in humans, we then performed a pilot study in a canine model, which is an accepted preclinical animal model for vascular surgery. Clinically relevant-sized TEVG implants (4 mm diameter and 7 cm length) were grown from canine SMC and decellularized. The HA-coated and uncoated grafts were implanted end-to-end in canine carotids (Figure 5a). HA-coated (\( n = 3 \)) TEVG were implanted on one side, and uncoated TEVG (\( n = 3 \)) controls were implanted contralaterally. The animals received aspirin as the sole anticoagulant for the duration of this study.

Grafts were explanted after 5 weeks, and the coated TEVGs demonstrated differences over the uncoated TEVGs. A gross image in Figure 5b shows aggressive thrombus formation (complete blood flow occlusion) in the uncoated TEVG, and angiographic absence of blood flow prior to explantation (Figure 5h). Conversely, the coated TEVG was totally patent (Figure 5c), and allowed blood flow preservation (Figure 5i), as demonstrated by angiogram imaging prior to explantation. Quantitative H&E image analysis indicated a five-fold percentage of patent lumen area of the coated grafts as compared to uncoated (Figure 5d, \( n = 3, ****p < 0.0001 \)). The thrombosis and neointima reduced

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**Figure 5.** Canine carotid implants. Uncoated and hyaluronic acid (HA) coated decellularized tissue engineered vascular grafts (TEVGs). a) Gross image of dog carotid TEVG implant of 4-mm inner lumen diameter graft. Cross image of explanted, b) uncoated and c) coated TEVGs after 5-week implantation, shown in cross-section. d) Percentage patent lumen area of the mid-graft of the uncoated and HA-coated TEVGs at 5 weeks. (\( n = 3, ****p < 0.0001 \), by two-way ANOVA with Bonferroni correction). e) Inner diameter and f) outer diameter of the uncoated and HA-coated TEVGs at 5 weeks. (\( n = 3, ****p < 0.0001, *p < 0.005 \), by unpaired, two-sided \( t \)-test with Welch’s correction). The lumen area of uncoated graft dramatically decreased with a small extent of dilation, while the coated graft kept a total patency and the original diameter. h) Angiogram imaging confirming absence of blood flow in the uncoated TEVG, while i) the HA-coated TEVG retained flow. The grafts are indicated between two black arrows, and the flow directions are indicated by the yellow arrows. g) Intact HA coating on the explanted TEVGs shown by Alcian blue stain.
the inner diameter of uncoated graft to less than 1 mm, while the coated graft kept the original size of the lumen area and diameter (Figure 5e, n = 3, ** p < 0.0001). The uncoated grafts were slightly dilated relative to coated grafts (Figure 4f, n = 3, \( \tau p < 0.05 \)). Following the explant, the intact and continuous HA coating on the coated TEVG was verified through the entire vessel by alcian blue staining at pH 2.5 (Figure 5g). The coating thicknesses of the explants were 2.5 ± 0.6 μm, showing 50% of the coating thickness remained during 5 week dog implantation. Some cells adherent on HA coating are shown by the pink nuclei on the coating.

Histological analysis corroborated the functional angiograms, where the uncoated TEVG had dense thrombus formation in 2 of 3 cases (Figure 6a,e). The HA-coated TEVG supported blood flow and histologically was thrombus free (Figure 6b,f). (A fresh clot containing isolated red cells formed during the harvest procedure (Figure 5S, Supporting Information). Neointimal formation was dense in the uncoated TEVG (Figure 6e) but absent in the coated TEVG (Figure 6f,g). Furthermore, the coated TEVG demonstrated a partial endothelialization on the coating both at the anastomosis and mid-region of the grafts (Figure 6f,g,j,k). In contrast, the uncoated TEVG did not demonstrate EC lining anywhere along the graft length (Figure 6i). The HA coating was compatible with EC adhesion and spreading, as visible by SEM, while the uncoated TEVG showed adhesion of red cells and platelets (Figure S6, Supporting Information). \( \alpha \)-SMA positive SMCs from the native arteries also migrated into the anastomosis and mid-region of the graft wall (Figure 6i–k).

For both the uncoated and coated TEVGs, we stained the vascular wall for collagen IV (Figure 6m–p) and found well-wide spread expression of collagen IV on the HA-coated TEVG walls, and less expression on the uncoated control. We also stained the vascular wall for collagen I and III stained by Sirius red (Figure 6q–t) and found concentric deposition of both collagen types within the coated TEVG infiltrating transmurally from the adventitial-like tissue layer into TEVGs. The noncoated groups had luminal thrombus formation indicating a well-formed thrombus and absence of remodeling. Figure S7, Supporting Information, presents Collagen IV and Sirius red stains of the preimplant as a comparison.

3. Discussion

Currently, there are no clinically available small-diameter vascular grafts that do not succumb to thrombosis at unacceptable high rates upon implantation. To date, no surface coating or cell-based approach has demonstrated a significantly improved clinical outcome. A decellularized TEVG has been preliminarily evaluated for safety and function in the hemodialysis setting, where grafts are 6 mm in diameter and blood flow rates are >1.0 L min\(^{-1}\).\(^{[35]}\) These grafts appear safe and durable, and remodel with cells from the host over time. However, these decellularized TEVGs\(^{[29]}\) suffer primary patency rates that are similar to ePTFE grafts used in this setting, implying that acute thrombosis remains a functional issue.

The goal of our approach was to prevent thrombogenic graft failure at small diameters, by masking luminal collagen with a chemical coating that effectively shields platelets from collagen-triggered activation, while encouraging endothelialization. We also endeavored to attach this coating using covalent chemistry that was not complicated by inadvertent cross-linking of the collagenous matrix in the graft wall, since decellularized engineered grafts normally undergo remodeling by the host after implantation that might be impeded by the presence of artificial cross-links within the extracellular matrix.\(^{[39]}\)

We postulated that a carefully designed HA coating that supports endothelialization, while shielding collagen from platelets, would successfully accomplish these goals.\(^{[31]}\) The HA coating was bound onto the collagenous graft by preactivating the amine groups in maleimide groups using a bifunctional cross-linker. Maleimide’s preferential reaction with sulfhydryl groups (that were present on functionalized HA) prevented nonspecific cross-linking reactions within the vascular wall, as thiol groups are vanishingly rare in the collagen of the vascular wall. Thus, we have developed an ≈5 μm thick gel-like coating that preserved high hydration, much like the native glycocalyx.

In the ongoing remodeling of decellularized, engineered biological grafts, SMCs infiltrate into the graft wall.\(^{[39,41]}\) Some previous studies of cross-linked grafts that were treated with EDC,\(^{[38]}\) glutaraldehyde, or genipin\(^{[37]}\) showed that SMCs could not migrate into the graft wall, contributing to gradual dilatation over time in vivo. We chose NHS–PEG\(^{2-}\)–maleimide as a cross-linker, so that the active esters could be easily reacted with amine groups, which are a dominant reactive component in graft. Maleimide, as the other end of the cross-linker, covalently reacted largely with thiol-modified HA. This design avoided a traditional amine-to-carbonyl cross-linking chemistry method, which may lead to hardening of the graft and impaired remodeling due to covalent collagen cross-linking. Here, the amine-to-thiol cross-linking chemistry assured a limited number of reactions within the graft wall, since the incidence of unreacted thiol groups in collagen is much less than carboxyl groups.

Our findings using in vitro blood tests showed that the HA coating to be strongly hydrophilic and with a smooth surface, which may contribute to low fibrinogen adsorption, platelet adhesion, and fibrin formation. Furthermore, we found that the HA coating provided excellent antithrombogenic functionality, and supported reendothelialization in small and large animal models on different decellularized vascular grafts (native rat aorta and TEVG). The coated TEVG showed preservation of functional blood flow, which was diminished or lost in the control, uncoated TEVG implants. EC lining and covering the graft were found in the mid-graft region densely populating the surface as well as at anastomotic regions.

In particular, the dog is relatively hypercoagulable, meaning this is a much more aggressive functional model than sheep or pigs.\(^{[35]}\) For instance, the canine platelet aggregation sensitivity to ADP is low relative to humans, and inhibition of aggregation by prostaglandin D2 is weak.\(^{[35]}\) Furthermore, the fibrinolytic system is more active in dogs, and dogs have significantly higher concentrations of fibrinogen relative to humans. As a result, collagen-induced thrombosis formation and clotting time are more aggressive in canine species over humans.

Some limitations exist for this study, however. The first is the low number of implants in the dogs, and the limited duration of follow-up, which future studies will address. In addition,
the long-term impacts of the HA coating on intimal hyperplasia and other remodeling in a large animal species would be informative. Future studies might include an implant in a less aggressive preclinical model that would be more human-predictive, such as the baboon. This work shows functionality of this covalently bound HA coating for small-caliber arterial grafting, and lends support for future, larger scale studies.

4. Conclusion

To mimic the antithrombogenic surface of native vessels, in this work a HA-modified surface was established on the lumen of decellularized TEVG. This layer effectively shields blood platelets from collagen-triggered activation, while allowing endothelial repopulation over time in rats and dogs. Hence, HA-coated
5. Experimental Section

Culture of TEVG: The bioreactor systems, cell seeding, and medium replenishment proceeded as described previously. Briefly, the degradable vessel scaffold was fabricated by sewing a polyglycolic acid (PGA) mesh (4 mm diameter, 18 cm long, 1 mm thickness, Biomedical Structures) with Dexon suture (Synteres) around a deformable silicone tube (Saint Gobain). A suspension of passage three dog aortic SMCs, 12 million in total for making one vascular graft, was seeded onto a PGA mesh surface and quickly infiltrated the entire mesh in a minute. Two TEVGs were cultured in 1.5 L of high glucose Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, USA) with bovine serum (20% for 0–4 weeks and 10% 5–8 weeks) (Fisher, USA). 10 ng mL−1 basic fibroblast growth factor (R&D systems), 10 ng mL−1 platelet-derived growth factor (R&D systems), 3 ng mL−1 transforming growth factor beta 1 (PeproTech), 3 ng mL−1 copper sulfate (Sigma), 50 ng mL−1 proline (Sigma), 50 ng mL−1 glycine (Sigma), 20 ng mL−1 alanine (Sigma), 50 ng mL−1 ascorbic acid (Sigma), 100 U mL−1 Penicillin G (Sigma), and 1 g L−1 lactic acid (Sigma). TEVG were cultured under pulsatile conditions in the bioreactor at 37 °C, 5% CO2 for 8 weeks. Then the vessels were decellularized by using a high pH salt solution (H2O, 1 M NaCl, 0.1 M NaOH, 25 × 10−3 M EDTA) for 4 h, Benzonase solution (2 U mL−1) for 6 h, 0.3% CHAPS (Sigma), 1 M NaCl (Sigma), 0.1 M NaOH (Sigma), and 25 × 10−3 M EDTA (Boston Bioproducts) for 4 h, and extensive rinsing in PBS. All decellularization steps were carried out at room temperature with 200 rpm agitation under sterile conditions.

Rat Aorta Harvest: All experimental animal work (protocol number: 2019-11190) was approved by the Yale University Institutional Animal Care and Use Committee. Thoracic aortas were harvested from young adult (3-month-old) male Sprague–Dawley rats. After induction of anesthesia with sodium pentobarbital, a transverse incision was made below the costal margin, entering the abdominal cavity. The heart and aorta were exposed after resecting the ribs. The portion of the aorta at the level of the thoracic aorta to the renal arteries was excised along its longitudinal axis, retrieved, and placed in PBS. The retrieved aortas were of approximately 1.0–1.5 mm inner diameter, and 20 mm length.

Decellularization of Rat Aortas: Individual rat aortas were rinsed in PBS three times for 10 min each. The vessel was then placed in the first decellularization solution containing 8 × 10−3 M CHAPS, 25 × 10−3 M EDTA, and 1 M NaCl in PBS at 37 °C overnight. After PBS rinsing, the vessel was then placed in the second decellularization solution containing 1.8 × 10−3 M SDS (Sigma), 1 M NaCl, and 25 × 10−3 M EDTA in PBS at 37 °C overnight. The vessel was incubated with shaking in 20% PBS in PBS with 1% Anti-Anti (Sigma) overnight 37 °C to remove DNA. The vessel was rinsed in PBS three times for 2 h each. Decellularized grafts were then stored in sterile saline at 4 °C until use, for coating, characterization, or in vivo implementations.

Coating of Decellularized Grafts with HA: All solutions were perfused via a custom-built perfusion bioreactor, where the decellularized grafts (either TEVGs, or native rat aortas) were mounted end-to-end with two chamber arms, and solutions were injected into a closed loop system that was continuously circulated via a peristaltic pump (Figure S2, Supporting Information). As illustrated in Figure 1, succinylmaleimidopropionamido-diethylene glycol (NHS–PEG2–maleimide, Thermo scientific) (2) was dissolved in PBS buffer at 5.5 mg mL−1 and perfused over decellularized vessel or TEVG for 30 min (1), thereby modifying the exposed primary amines with maleimide groups, which are thiol-reactive (3) (Figure 1a). Covalent attachment of thiol-functionalized hyaluronan (4) onto the thiol reactive vessel/graft (3) was accomplished by incubating the grafts in the presence of thiol-modified sodium hyaluronate (HA, Glycosil, ES1-BIO) with a molecular weight of 240 ± 30 kDa that was dissolved in distilled water at 10 mg mL−1 (Figure 1b). The thiolated HA was continuously perfused over the vessel/graft for 1 h, thereby allowing both reaction of the thiolated HA with the functionalized vessel/graft surface (5), and also allowing disulfide bridge formation of the HA to form a thin and uniform layer on the lumen of the vessel/graft (6) (Figure 1c).

Quantification of Amine Groups on Graft Surface: We quantified the accessible amine groups on the collagenous graft luminal surface (attachable ends or “hooks”, Figure S3a, Supporting Information) on decellularized TEVGs and rat aortae using amine-reactive iron nanoparticles that are easily quantifiable via SEM with X-ray spectroscopy (SEM–EDX).

Structural Characterization: The HA coating architecture on the TEVG samples was examined via TEM, SEM (model S-4700, Hitachi) and toluidine blue histology staining. TEM samples were fixed in 2.0% paraformaldehyde and 2.5% glutaraldehyde containing 0.075% ruthenium red, 75 × 10−3 M lysine, and 0.1 M cacodylate at pH 7.2 and then fixed in the same solution with lysine removed. Following a rinse with 0.1 M cacodylate alone, samples were post-fixed with 1% osmium tetroxide to enhance contrast, dehydrated through a graded series of ethanol solutions, and embedded in LIX112 Resin. Samples were viewed on a FEI Tecnai Biowin TEM at 80 kV. Representative images were taken using a Morada CCD digital camera using iTEM software (Olympus). SEM samples were rinsed with PBS for 30 min, fixed with 2.5% glutaraldehyde for 15 min and dehydrated through a series of graded alcohol solutions, each solution for 15 min. The specimens were air-dried overnight and the dry samples were sputter-coated with carbon and observed under the SEM at an accelerating voltage of 20 kV. For toluidine blue staining, samples were fixed in 10% (v/v) neutral-buffered formalin for 1 h, embedded in paraaffin, cut into 5 μm sections, stained with glycine, and then dehydrated using a graded series of ethanol solutions, and embedded in LIX112 Resin. Images were obtained using an Axiovert microscope (Zeiss, Thornwood, NY) equipped with AxioCam HR.

Surface Energy: Surface contact angles were measured by a drop shape analysis system DSA100 (Krüss, Hamburg, Germany) at room temperature followed by image processing of a sessile drop of 5 μL of test distilled water and diiodomethane (CH2I2) with DSA 1.8 software. The Fowkes theory(6) was applied (Equation 1) to calculate the polar (γP) and dispersive (γD) components of the surface energy for each surface: γL and γS were related to the liquid surface tension and solid surface energy, respectively.

$$\gamma = \gamma_L + \gamma_S = \gamma_L + \gamma_S = 2\sqrt{\gamma_L \gamma_S} \left(1 + \cos \theta\right)$$

Mechanical Characterization: Mechanical tests were performed on uncoated (controls) and HA-coated decellularized TEVGs using an Instron 5848. Grafts were longitudinally cut into strips (0.5 mm thickness × 6 mm width × 45 mm length) for mechanical analyses. Tissue strips were cyclically preconditioned for 3 cycles to 15% strain, and then pulled until failure at a strain rate of 1% s−1. The ultimate strength and Young’s modulus were calculated.

Assessment of HA Coating Stability: HA coating was enzymatically digested and quantified via both a carbazole assay, and using LC–MS (Agilent 1200 LC-MS, Agilent Technologies, Inc., Wilmington, DE). Digestion buffer was composed of 390 units of collagenase type I, and 15 units of hyaluronidase (both Sigma) dissolved into 2 mL of PBS at pH 6. HA-coated TEVGs were incubated in digestion buffer at 37 °C for at least 4 h.

The digested coating on the decellularized TEVGs was quantified using the carbazole assay. Briefly, 40 μL of buffer retrieved from the incubated samples was added to 200 μL of 0.025 M sodium tetaborate with a pH of 9.5 and heated to 100 °C for 15 min followed by the addition of 8 μL of carbazole solution (0.125% carbazole in ethanol). Absorbance was measured at 530 nm.

LC–MS combined with disaccharide compositional analysis was used to validate the carbazole assay. Briefly, following enzymatic digestion,

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Fibrin Formation: Whole human blood was premixed with CaCl$_2$ (8 × 10$^{-3}$ M, Sigma) and thrombin from human plasma (0.25 U mL$^{-1}$, Sigma), then 20 μL of mixed blood immediately was put on the flat surfaces of the uncoated and HA-coated TEVGs for 15 min at 37 °C until a coagulation and gel formation had occurred. The samples were gently rinsed with PBS for three times, fixed with 2.5% glutaraldehyde for 15 min, dehydrated with a series of graded alcohol solutions, and imaged via SEM. The amount of adherent fibrin polymer was quantified. The number of biological replicates was three (N = 3).

Thrombin generation: 300 μL of whole human blood containing 3.2% sodium citrate was loaded into 2.5 mm long, 6 mm diameter grafts. Two ends of the graft were clamped by vein clamps (Figure S8, Supporting Information), and the grafts with the blood were incubated for 3 h at 37 °C. Thereafter, the blood was collected and centrifuged at 3000 rpm for 15 min. ELISA using a commercial kit for thrombin F1+2 fragment (CUSABIO, catalog number CSB-E0982H2) was performed after the incubation period according to manufacturer’s instructions. The number of biological replicates was three (N = 3).

Macrophage Adhesion: Peritoneal macrophages from adult male C57BL/6 mice were harvested by peritoneal lavage 4 days after intraperitoneal injection of thioglycollate (3% w/v). After washing cells in RPMI 1640 supplemented with 10% FBS, 100 units mL$^{-1}$ penicillin and 100 units mL per 1 streptomycin. After 4 h, nonadherent cells were washed away, and macrophages were incubated in fresh medium containing DMEM, 10% FBS, and 20% L929 cell-conditioned medium for 24 h, and cells were maintained in culture as an adherent monolayer.[25] Macrophages with the density of 4 × 10$^6$ cells cm$^{-2}$ were seeded onto the flat surfaces of the uncoated and HA-coated TEVGs. The samples were cultured in DMEM containing 10% FBS, 10% L929-conditioned medium, and 90 U mL$^{-1}$ hyaluronidase for 2 and 24 h. For immunofluorescence, cultured cells were fixed with 4% paraformaldehyde for 15 min, then permeabilized and blocked with PBS containing 5% BSA and 0.2% Triton X-100 for 1 h, and subsequently incubated in primary antibody CD68 (Bio-Rad, catalog number: MCA1957GA, dilution 1:600). After washing cells in PBS, secondary antibody Alexafluor 555 (Invitrogen, dilution 1:500) was applied for 1 h, and nuclei stained with DAPI for 1 min at RT. Digital images were captured on an Olympus IX71 inverted microscope. The average number of adhered macrophages on the surfaces of HA-coated and noncoated TEVGs was counted by Image J morphometric analysis. The number of biological replicates was three (N = 3).

In Vivo Functional Assessment of HA Coatings in a Rodent Model: Uncoated and HA-coated decellularized rat aorta (1.0–1.5 mm inner diameter, 10–20 mm length) were implanted in the abdominal aorta of ≈12-week-old Sprague–Dawley rats weighing ≈300 g using an end-to-end anastomosis (n = 6 rats for each group). Uncoated and HA-coated decellularized rat aorta went through a strict sterile process during the harvest, decellularization, and coating procedures. All procedures were performed according to a Yale University IACUC approved protocol. The surgical model involved a midline laparotomy and subsequent exposure of the infrarenal abdominal aorta. No antiplatelets were utilized for this procedure. Microclamps were used to halt blood flow, followed by the removal of a 15 mm segment of aorta that was then replaced by an end-to-end anastomosed graft using 8-0 monofilament polypropylene sutures. The distal, and subsequently the proximal vascular clamps were slowly removed, and flow was restored through the graft. Total ischemia time was 30 to 60 min. Blood flow was monitored using a Doppler ultrasound imaging at 30 days. Data was expressed as mean (n = 6 rats per group) ± SEM (standard error of mean). Unpaired, two-tailed Student’s t-tests were performed to evaluate whether the different groups were significantly different from each other in their functional assessments. A value of p ≤ 0.05 was considered statistically significant. Rat explants were analyzed using the following stains: H&E (Fisher Chemical) for general evaluation, Elastic -Van Gieson for elastic fiber, and immunostaining for primary α-SMA (Dako, catalog number:M0851, dilution 1:10000) with the secondary antibody Alexafluor 555 (Invitrogen, dilution 1:500) for SMCs, vWF (Santa Cruz, catalog number:sc-30668, dilution 1:50) with the secondary antibody Alexafluor

the HA disaccharides were recovered by centrifugal filtration using an YM-10 spin column and lyophilized overnight. All disaccharide standards were purchased from Didorun Ltd (Manchester). Unadsorbed disaccharides were then labeled using 2-aminoacridine (AMAC) by adding 10 μL of 0.1 M AMAC solution. Next, 10 μL of 1 M NaH$_2$CO$_3$ was added to the reaction mixture and incubated at 45 °C for 4 h. Finally, the AMAC-tagged disaccharides were diluted to different concentrations (0.5–100 ng) using 50 vol% aqueous DMSO. LC–MS analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Inc. Wilmington, DE) equipped with a 3600 ion trap and a binary pump. A Poroshell 120 C18 column (3.0 × 150 mm, 2.7 μm, Agilent) at 45 °C was used. Eluent A was 80 × 10$^{-3}$ mol ammimunum acetate and eluent B was methanol. Eluent A and 15% eluent B were flowed (150 μL min$^{-1}$) through the column for 5 min, followed by linear gradients of solution B (15–30%) from 5 to 30 min. The electrospray interface was set in negative ionization mode, with a skimmer potential of −40.0 V, a capillary exit of 40 V and a source temperature of 350 °C, to obtain the maximum abundance of the ions in a full-scan spectrum (300–1200 Da). Nitrogen (8 L min$^{-1}$, 40 psi) was used as a drying and nebulizing gas.

HA Coating Stability in Cell Culture Medium with 90 U mL$^{-1}$ Hyaluronidase: In order to ascertain the stability of HA on the decellularized TEVGs in long-term culture, HA eluted into cell-culture medium was quantified for 2 week exposure. Briefly, uncoated and HA-coated decellularized TEVGs of 5 mm length and 4 mm diameter were placed in 24-well plates at 37 °C in medium containing DMEM, 10% FBS, and 90 U mL$^{-1}$ hyaluronidase. At selected time points (0, 1, 3, 5, 7, and 14 days), 1 mL of incubation medium was removed and replaced with new medium. Coated TEVG samples were removed at selected time points, and the coating thickness assessed using the toluidine blue staining, coupled with Image J morphometric analysis. The number of biological replicates was three (N = 3).

HA Coating Stability in Blood Plasma: HA-coated TEVGs of 5 mm length and 4 mm diameter were incubated in fresh blood plasma for 2 weeks. Whole blood from Sprague–Dawley rats was drawn into a syringe containing 3.8% sodium citrate (volume ratio blood: sodium citrate 9:1). Whole blood was centrifuged at 3000 rpm for 15 min to isolate the platelet-poor plasma (PPP). PPP directly added to the samples in 24-well plates and incubated at 37 °C. The plasma was replaced every morning for 14 days. Coated TEVG samples were removed at selected time points (1, 7, and 14 days), and the coating thickness assessed using the toluidine blue staining, coupled with Image J morphometric analysis. The number of biological replicates was three (N = 3).

Platelet and Whole Blood Adhesion: Whole blood from a human donor was drawn into a vacutainer containing 3.2% sodium citrate (volume ratio of blood: sodium citrate, 9:1) (BD), and centrifuged at 1500 rpm for 15 min to isolate the platelet-rich plasma (PRP). Whole blood or PRP was then incubated for 30 min at 37 °C on the flat surface of uncoated and HA-coated decellularized TEVGs with 6 mm diameter round pieces. To observe the morphology of the platelets and red cells, the samples were fixed with 2.5% glutaraldehyde for 15 min, dehydrated with a series of graded alcohol solutions, and imaged via SEM. To quantify the number of adhered platelets, the samples were fixed with 4% paraformaldehyde and then stained by rhodamine phallidin (Invitrogen, catalog number: R415, 1:200). The number of biological replicates was three (N = 3).

Fibrinogen Adsorption: Uncoated and HA-coated TEVGs with 6 mm diameter round pieces were immersed in 50 μg mL$^{-1}$ of fibrinogen from human plasma. Alexa Fluor 488 conjugated (Thermo fisher, catalog no. F31919) in PBS for 1 h, then rinsing three times in PBS and imaging by fluorescence microscopy. Fluorescence intensity of the adsorptive fibrinogen was measured by Image J. The number of biological replicates was three (N = 3).
488 (Invitrogen, dilution 1:500) for ECs, and CD45 (Abcam, catalog number:ab10558, dilution 1:200) with the secondary antibody Alexafluor 488 (Invitrogen, dilution 1:500) and CD68 (Bio-Rad, catalog number: MCA1957G, dilution 1:600) with the secondary antibody Alexafluor 555 (Invitrogen, dilution 1:500) for inflammatory markers associated with leukocyte and macrophage infiltration.

In Vivo Functional Assessment of HA Coatings in a Canine Model: To assess the coating functionality in a relevant preclinical model, the TEVGs were implanted end-to-end within the carotid arteries of the dog bilaterally, with one side control (n = 3 dogs) and the other HA coated (n = 3 dogs). Uncoated and HA-coated decellularized TEVGs went through a strict sterile process during the fabrication, decellularization, and coating procedures. All procedures were performed according to a Synchrony Labs Institutional Animal Care and Use Committee (IACUC) approved protocol. Three 1-year-old Mongrel Hound dogs with weighing 25–30 kg were used for canine implantations. The animals were anesthetized and 7 cm long, 4 mm inner diameter grafts were implanted into the carotid arteries using standard vascular techniques. Heparin was administered intravenously (110 U kg−1) to each animal before application of the vascular clamp to the recipient artery. The animals were given one aspirin (325 mg) daily post-surgery for the duration of the study. The blood flow of all implants was verified using an angiogram prior to explanation, and all grafts were explanted at 5 weeks. Data were expressed as mean (of 3 dogs per group) ± SEM (standard error of mean). Unpaired, two-tailed Student’s t-tests were performed to evaluate whether the groups were significantly different from each other in the functional assessments. A value of p ≤ 0.05 was considered statistically significant. Dog explants were analyzed using the following stains: alcan blue staining at pH 2.5 for HA coating, H&E for general evaluation, Anti-Collagen IV antibody (Abcam, catalog number: ab6586, dilution 1:200) for collagen IV, Sirius red (Abcam, catalog number: ab150683) for collagen, immunostaining for SMCs (α-SMA, Dako, catalog number:M0851, dilution 1:1000), and ECs von Willebrand factor (vWF, Dako, catalog number: A0082, dilution 1:200), and SEM as described above.

Statistical Analysis: Statistical significance (P) was determined with unpaired, two-sided t-test with Welch’s correction and two-way ANOVA with Bonferroni correction as indicated in each figure legend. Data are expressed as mean ± standard deviation (±SD).

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

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Conflict of Interest
Prof. L. E. Niklason is a founder and shareholder in Humacyte, Inc, which is a regenerative medicine company. Humacyte produces engineered blood vessels from allogeneic smooth muscle cells for vascular surgery. Prof. Niklason’s spouse has equity in Humacyte, and Prof. Niklason serves on Humacyte’s Board of Directors. She is an inventor on patents that are licensed to Humacyte and that produce royalties for her. She has received an unrestricted research gift to support research in her laboratory at Yale. Humacyte did not fund these studies, and Humacyte did not influence the conduct, description, or interpretation of the findings in this report.

Authors Contributions
S.D. and J.W. contributed equally to this work. S.D. and J.W. conceived the study, designed, and optimized the experiments, analyzed the data, and prepared the manuscript. T.L., A.W., H.B., C.C., N.K., A.C., S.S., K.Y., W.W., J.M., L.Z., and M.H.K. performed the experiments and analyzed the data. L.Q. and G.L. conducted the rat surgery. A.K. conducted the dog surgery. T.R.K. advised experimental design. R.J.L. designed the chemical reaction of coating. L.E.N. mastered the mind guidance, obstacle removal, and interpretation of results.

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