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Click-coated, heparinized, decellularized vascular grafts

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ABSTRACT

A novel method enabling the engineering of a dense and appropriately oriented heparin-containing layer on decellularized aortas has been developed. Amino groups of decellularized aortas were first modified to azido groups using 3-azidobenzoic acid. Azide-clickable dendrons were attached onto the azido groups through "alkyne–azide" click chemistry, affording a tenfold amplification of adhesions sites. Dendron end groups were finally decorated with end-on modified heparin chains. Heparin chains were oriented like heparan sulfate groups on native endothelial cells surface. X-ray photoelectron spectroscopy, nuclear magnetic resonance imaging, mass spectrometry and Fourier transform infrared FTIR spectroscopy were used to characterize the synthesis steps, building the final heparin layered coatings. The continuity of the heparin coating was verified using fluorescent microscopy and histological analysis. The efficacy of heparin linkage was demonstrated with factor Xa anti-thrombogenic assay and platelet adhesion studies. The results suggest that oriented heparin immobilization to decellularized aortas may improve the in vivo blood compatibility of decellularized aortas and vessels.

1. Introduction

Demand for non-thrombogenic arterial conduits persists due to the poor clinical performance of existing synthetic grafts for small-diameter artery applications. Decellularized native and tissue-engineered vascular grafts have shown success in large-diameter vascular applications [1–4]. The decellularization process necessary for the removal of cellular antigens also removes the endothelial cells (ECs) lining the lumen that is responsible for inhibiting coagulation and platelet adhesion [5]. Following the removal of ECs, the damaged vascular wall contains exposed collagen, a highly thrombogenic surface, which in small-diameter vascular grafts activates platelets and blood coagulation proteins [6]. Thus, one potential solution to preventing thrombus formation, subsequent occlusion and graft failure is to modify the decellularized vascular surfaces by "hiding" the exposed thrombogenic collagen with non-thrombogenic chemical structures such as heparan sulfate [7,8].

To avoid thrombogenic failure, the common approach used by others in the past has been the linking of heparin to decellularized grafts. Typically, heparin is linked to the extracellular matrix (ECM) using a "one-to-one" (one active group of ECM to one heparin chain) crosslinker such as glutaraldehyde [9] or 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) [10–12]. As there are few surface accessible amino groups on the ECM, the one-to-one approach results in a low surface coverage of heparin. In addition, heparin's anticoagulant efficacy depends on its ability to bind antithrombin III (ATIII) through the appropriate presentation of its ATIII-specific pentasaccharide sequence [13]. Lack of control over the heparin orientation, in combination with small amounts of heparin attachment, have been the main reasons for limited success following chemical immobilization of heparin on vascular grafts. These issues have been somewhat addressed on polymeric grafts, where the surface modification methodologies are more numerous, with end-grafted heparin layers currently being clinically favored [7,14–16]. As an example of heparin-coated vascular grafts, Gore's end-on heparin-coated Dacron grafts, Propaten®, demonstrated, in a femoroepopliteal bypass clinical study, a 17% increase in first-year patency values, as compared to untreated polytetrafluoroethylene (PTFE) grafts [17]. However,

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the primary patency rate of Propaten® grafts at 48 months was 17.2% lower than in ipsilateral autologous saphenous vein (the industry gold standard) [18].

While directional coupling chemistry has been used to immobilize heparin to polymeric structures such as PTFE[19] and nanomaterials [2–23], it has not been previously reported on dendrons/dendrimers or on biological structures. Here we present a novel method that enables the synthetic engineering of a dense, oriented heparin-containing layer on biological structures, such as decellularized vessels. We are able to recreate the dense packing and relevant structural orientation by first amplifying the existing labile chemical groups using azide-clickable dendrons and then decorating the surface of the dendrons with end-on oriented heparin, which mimics the orientation of heparan sulfate on the EC surface.

2. Materials and methods

2.1. Materials

Polyester-8-hydroxyl-1-acetylene bis-MPA dendron, 3-mercaptopropionic acid, 3-aminobenzoic acid, trityl chloride, adipic acid dihydrazide (ADH), EDC, *N*-hydroxysuccinimide (NHS), 4-dimethylaminopyridine, 2-morpholinoethane sulfonic acid, trypsin, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), phosphate-buffered saline (PBS), $NaBH_3CN$, NaCl, general solvents and reagents were purchased from Sigma. Sulfosuccinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Thermo Fisher Scientific Inc. Fetal bovine serum (FBS) was purchased from Gibco. Factor Xa (FXa) and ATIII were purchased from Sigma.

2.2. Preparation of decellularized aortic tissues

Fresh porcine descending abdominal aortas (inner lumen diameter ~ 2 cm) were harvested from a local slaughterhouse and transported on ice to the laboratory. Immediately after arrival, the aortas were cleaned of adherent tissues and fat, and rinsed in PBS. The aortas were decellularized by first incubating them in a solution of CHAPS, 1 M NaCl and 25 mM EDTA in PBS for 24 h on a stir plate at 37 °C. The aortas were then washed three times in PBS for 10 min, followed by an overnight rinse in 10% FBS in PBS for 24 h on a stir plate at 37 °C. After the decellularization process, using a sterilized cutting tool, aortas were cut into 1 cm discs. To verify the removal of all cellular materials, the aortas were fixed in neutral-buffered formalin, then stained with hematoxylin and eosin (H&E) to identify the nuclear material. To evaluate the decellularization quantitatively, the DNA content of fresh and decellularized porcine aortas was determined using the PicoGreen assay. Briefly, aorta segments were lyophilized, weighed and digested in papain buffer (papain 125 μg ml^{-1} (Sigma, Saint Louis, MO), 5 mM cysteine–HCl, and 5 mM disodium ethylenediaminetetraacetic acid (EDTA) in PBS) at 60 °C overnight. The papain sample solution was diluted with buffer (10 mM Tris–HCl and 1 mM EDTA, pH 7.5; Invitrogen) and incubated with an equal volume of Quant-iT™ PicoGreen® dsDNA reagent (Molecular Probes, Eugene, OR), then, using a fluorometer, the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The complete decellularization of the aortas was also confirmed using β-actin immunoblotting. Proteins were extracted from homogenized frozen fresh and decellularized porcine aortas tissue samples in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 vol.% Triton X-100, 0.5 wt./vol.% sodium deoxycholate and 0.1 wt./vol.% SDS) containing freshly added proteinase inhibitors (Sigma–Aldrich, St. Louis, MO). The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Samples were prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by boiling 30 μg of proteins in SDS sample buffer containing 2% β-mercaptoethanol (final concentration) for 5 min. SDS–PAGE and immunoblotting were performed to verify complete aorta decellularization. Briefly, proteins were separated by electrophoresis, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and immunoblotted with mouse monoclonal antibody to β-actin (1:2500; Sigma–Aldrich), followed by horseradish peroxidase-conjugated goat anti-mouse secondary antibody. Blots were developed using enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL). The DNA content and β-actin immunoblots are shown in Supplementary Fig. 1. The absence of H&E cellular nuclei staining was taken as indicative of complete decellularization of the aortas. Aorta pieces were then snap frozen at −80 °C and sliced to 35 μm thickness using a cryostat without using mounting medium, to preserve the native decellularized chemistry of the aortic luminal surfaces.

2.3. Synthesis of maleic attached heparin

Sulfhydryl-reactive end-on modified heparin was produced by end-on modifying heparin with maleimide, as illustrated in Fig. 1A. Briefly, heparin (100 mg, 8.3 μmol) was added to 10 ml of heated formamide, followed by the addition of dissolved ADH (100 mg, 920 μmol), and maintained at 50 °C for 6 h. Aqueous sodium cyanoborohydride (9.5 mg, 150 μmol) was added and incubated at 65 °C for an additional 24 h. The reaction mixture was diluted with 50 ml of water and dialyzed against 2 l of water for 48 h using a 3500 molecular weight cutoff (MWCO) dialysis membrane. The retentate was recovered, lyophilized and purified by ethanol precipitation to obtain the ADH–heparin derivative (Fig. 1, compound **2**). Sulfo-SMCC (23 mg, 50 μmol) was added to the ADH–heparin derivative (75 mg, 5 μmol) in PBS buffer, and the solution was incubated for 2 h. Unreacted maleimide groups of sulfo-SMCC were quenched by aqueous solution of mercaptoethanol (1 mmol). The reaction solution was diluted with 10 ml of water and dialyzed against 2 l of water for 48 h using a 3500 MWCO dialysis membrane. The retentate was recovered, lyophilized and purified by ethanol precipitation to obtain the maleic–heparin derivative. The resulting compound of this three-step modification is identified in Fig. 1A as compound **3**.

2.4. Synthesis of heparin-modified dendron coatings of decellularized aortas by click chemistry

Pre-activated 3-azidobenzoic acid was used to modify the exposed primary amines of the decellularized aortas to azide groups. 3-Aminobenzoic acid (**4**) was converted to the corresponding azide (**5**) upon treatment with sodium azide and sodium nitrate under acidic conditions (Fig. 1B). Compound **5** (25 mg, 0.07 mmol) was then dissolved in *N*,*N*′-dimethylformamide (DMF, 1 ml), added to a solution of EDC (19.2 mg, 0.10 mmol) and stirred for 15 min at room temperature. Decellularized aortas (**10**) were then treated with this solution and incubated for 12 h at room temperature to afford azide-functionalized vascular grafts (**11**). The covalent attachment of **5** to decellularized aortas is presumably mediated by acylation of the amino groups in collagen (Fig. 1E, compound **11**).

Dendrons were then immobilized on the azido-modified decellularized aortas by "alkyne–azide" click chemistry [24]. For this, dendron **9** (14 mg, 7.88 μmol) and L-ascorbic acid (2.8 mg, 15.7 μmol) were dissolved in DMF (1 ml), added to the azide-containing decellularized aorta and exposed to $CuSO_4$ (0.1 M in PBS