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The blood and vascular cell compatibility of heparin-modified ePTFE vascular grafts

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Abstract

Prosthetic vascular grafts do not mimic the antithrombogenic properties of native blood vessels and therefore have higher rates of complications that involve thrombosis and restenosis. We developed an approach for grafting bioactive heparin, a potent anticoagulant glycosaminoglycan, to the lumen of ePTFE vascular grafts to improve their interactions with blood and vascular cells. Heparin was bound to aminated poly(1,8-octanediol-*co*-citrate) (POC) via its carboxyl functional groups onto POC-modified ePTFE grafts. The bioactivity and stability of the POC-immobilized heparin (POC–Heparin) were characterized via platelet adhesion and clotting assays. The effects of POC–Heparin on the adhesion, viability and phenotype of primary endothelial cells (EC), blood outgrowth endothelial cells (BOECs) obtained from endothelial progenitor cells (EPCs) isolated from human peripheral blood, and smooth muscle cells were also investigated. POC–Heparin grafts maintained bioactivity under physiologically relevant conditions *in vitro* for at least one month. Specifically, POC–Heparin-coated ePTFE grafts significantly reduced platelet adhesion and inhibited whole blood clotting kinetics. POC–Heparin supported EC and BOEC adhesion, viability, proliferation, NO production, and expression of endothelial cell-specific markers von Willebrand factor (vWF) and vascular endothelial-cadherin (VE-cadherin). Smooth muscle cells cultured on POC–Heparin showed increased expression of α -actin and decreased cell proliferation. This approach can be easily adapted to modify other blood contacting devices such as stents where antithrombogenicity and improved endothelialization are desirable properties.

Keywords

Vascular graft; Elastomer; Endothelial cell; Progenitor cell; Smooth muscle cell; Heparin; Hemocompatibility; Aminated poly(1,8-octanediol-*co*-citrate) (POC)

1. Introduction

Cardiovascular disease is a leading cause of death and morbidity in developed countries and patients diagnosed with this disease often require revascularization via bypass grafts to restore blood flow to tissue. Autologous vein bypass grafts, the gold-standard of care, cannot always be harvested, prompting the use of prosthetic vascular grafts. However, prosthetic vascular grafts have poor long-term patency, particularly when used in small diameter applications [1,2]. Therefore, there remains an urgent need for safe and effective materials for the fabrication of vascular grafts.

Heparin and other anticoagulants have been incorporated into biomaterials to inhibit intrinsic thrombogenicity [3–7]. Heparin immobilization strategies have included physisorption, electrostatic deposition, and covalent bonding to surfaces [3,4,6,8,9]. For example, cross-linked collagen surfaces have been used for the covalent immobilization of heparin to improve blood compatibility, but the use of highly thrombogenic materials such as collagen for vascular grafts may be problematic [4,10]. Other strategies include the controlled release of heparin from electrospun materials but soluble heparin can lead to increased risk of heparin-induced thrombocytopenia, which can be fatal in some circumstances [11]. To further augment vascular graft thromboresistance, some promising approaches have combined heparin immobilization with other known anti-clotting agents such as nitric oxide (NO) or thrombomodulin, but these multi-factorial approaches are complex and may be cost prohibitive for commercialization [6,9].

Our lab has reported the synthesis of citric acid-based biomaterials for use in tissue engineering applications [12–19]. In particular, poly(1,8-octanediol-*co*-citrate) (POC) is an elastomeric polyester that can be used to form composites, fabricated to have micro and nano-architectures, and used as a coating to modify medical devices to potentially improve their performance [14,16–21]. In previous studies by our group and others, POC was demonstrated to be biocompatible material with good hemocompatibility and minimal acute and chronic inflammatory responses *in vivo* [14,16,19,22]. POC also has been shown to support endothelialization under physiological flow conditions *in vitro* and *in vivo* [13,15]. Furthermore, the available carboxyl and hydroxyl groups on POC provide options to chemically modify the polymer network with macromolecules to engineer new functionality.

The quest to develop a functional vascular graft would benefit from a simple, effective and safe method to incorporate the antithrombogenic and anticoagulant properties of heparin into a biomaterial. In this regard, the effects of immobilized heparin on vascular cell processes should also be understood. Herein, we describe the functionalization of POC with heparin and investigate the effects of POC-immobilized heparin on whole blood clotting, platelet adhesion, and the adhesion, viability, and phenotype of endothelial cells and smooth muscle cells. As circulating progenitor cells can contribute to the endothelialization and arterial healing process, the effect of POC–Heparin on blood outgrowth endothelial cells (BOECs) is also investigated. BOECs are the progeny of bone marrow-derived circulating endothelial progenitor cells and emerging evidence supports their use in cell-based therapies for promoting postnatal vasculogenesis and clinical use in cardiovascular applications [23,24]. Differentiation of these cells may promote the formation of a healthy endothelium on

vascular graft surfaces [13,25,26]. This work also investigates whether immobilized heparin will have a negative effect on smooth muscle cell proliferation.

2. Materials & methods

2.1. Synthesis and preparation of POC

All reagents and chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless noted otherwise. The synthesis of POC has been previously described [19]. Briefly, equimolar amounts of citric acid and 1,8-octanediol monomers were melted under a flow of nitrogen gas at 160–165 °C and then the temperature of the system was lowered to 140 °C to create a pre-polymer. Subsequently, the pre-polymer was purified in water, freeze-dried and then reconstituted in ethanol. The pre-polymer was post-polymerized in tissue culture polystyrene (TCP) multiwell plates at 80 °C for 4 days. To fabricate POC-coated vascular grafts, thin-walled ePTFE tubes (6 mm inner diameter, 30–50 µm internodal distance, Zeus Inc., Orangeburg, SC) were coated with 10% pre-polymer using a previously described spin-shearing method and post-polymerized at 80 °C for 4 days [13]. The POC coating on the ePTFE was verified visually using SEM and toluidine blue dye, since it can bind with high affinity to negatively charged carboxylic acid functional groups [27].

2.2. Heparin immobilization to POC

POC samples were rinsed extensively in PBS at 37 °C for several days to remove unreacted monomers which may interfere with the conjugation chemistry. Prior to conjugation, POC-coated plates/vascular grafts were soaked in 0.1 M MES buffer (pH 5.6, containing 0.5 M NaCl) for 1 h. POC was covalently modified using standard carbodiimide chemistry. To covalently bind the carboxyl groups of POC to the diaminohexane intermediate, a solution of 50 mM DH solution was prepared with 300 mM *N*-(3 dimethylaminopropyl)-*N'*-ethylcarbodiimide (EDC) and 150 mM *N*-hydroxysuccinimide (NHS) catalysts in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, pH 5.6, containing 0.5 M NaCl and incubated with POC for 5 h at room temperature. The POC was extensively rinsed with 2 M NaCl and water to remove excess reagents. The immobilized DH was conjugated to the carboxylic acid groups of heparin by incubating the POC with heparin solution (2.5–5 mM) (heparin sodium salt isolated from porcine intestinal mucosa, MW15 kDa, 167 units/mg) with 100 mM NHS and 200 mM EDC catalysts in MES buffer overnight at room temperature. A concentration of 5 mM heparin solution was determined to be the maximal solution concentration during conjugation as determined previously by toluidine blue assay (data not shown) and used for blood compatibility and cell compatibility studies [28]. Excess reagents and non-covalently bound heparin were removed by extensive washing with 2 M NaCl solution and water. For cell compatibility studies, samples were sterilized by exposure to ethylene oxide gas.

2.3. Immobilized heparin quantification and contact angle measurements

The presence of heparin bonded to POC-coated ePTFE grafts was detected and quantified using the metachromatic dye, toluidine blue, as described previously [28]. The surface density of the POC–Heparin ePTFE coating was evaluated for stability after 14 days and 28 days of incubation *in vitro* at 37 °C in PBS and compared against freshly prepared samples.

To control for background dye binding, the POC–Heparin samples were compared against incubation-matched controls consisting of POC conjugated with diaminoethane. Static water-in-air contact angle measurements were taken over time using a custom-built contact angle goniometer (components from Rame-Hart, Inc., Mountain Lakes, NJ). A total of three different samples were measured for each surface type: ePTFE, POC–ePTFE and POC–Heparin ePTFE over a 15-min period.

2.4. Heparin detection by X-ray photoelectron spectroscopy (XPS)

Elemental analysis using X-ray photoelectron spectroscopy (XPS) was performed to verify the incorporation of diaminoethane and heparin by confirming the presence of nitrogen and sulfur peaks. POC–Heparin samples were prepared using 2.5 mM heparin solution during conjugation. XPS spectra were collected using Omicron ESCALAB (Omicron, Taunusstein, Germany) with a monochromated Al K α (1486.6 eV) 300 W X-ray source. Measurements consisted of broad survey scans as well as high-resolution N(1s) and S(1s) scans.

2.5. Isolation of blood outgrowth endothelial cells from human peripheral blood

The isolation and characterization of BOECs obtained from peripheral blood have been previously described by our lab [13]. Briefly, forty-five milliliters of peripheral blood were collected from adult volunteers in the presence of acid citrate dextrose (ACD, Solution A; BD Biosciences). All procedures involving blood collection were performed in accordance with the regulations of the Northwestern University Institutional Review Board. Peripheral blood mononuclear cells (PB-MNCs) were isolated from whole blood via histopaque density gradient centrifugation using Accuspin tubes (Sigma Aldrich, Milwaukee, WI). The isolated PB-MNCs representing the starting populations were suspended in endothelial cell (EC) growth medium-2 (Lonza, Baltimore, MD) without the fetal bovine serum supplement but with 5% allogeneic human serum (Lonza). The PB-MNCs were seeded onto fibronectin-coated plates (BD Biosciences; San Jose, CA), and cultured at 37 °C in a humidified incubator containing 5% CO₂. After 4 days, non-adherent cells were removed by complete media change, and media was changed every 3–4 days thereafter. BOECs were detected as tightly packed colonies with the characteristic cobblestone morphology of endothelial cells. After colony isolation, BOECs were expanded onto TCP and maintained through eight passages.

2.6. Bioactivity and antithrombogenicity assessment of POC–Heparin ePTFE grafts

2.6.1. Re-calcified whole blood clotting—The anti-clotting properties of POC–Heparin surfaces were assessed using modified re-calcified plasma and whole blood clotting assays [13,15]. Whole blood was collected from adult volunteers into ACD anticoagulant (BD Biosciences, Franklin Lakes, NJ). Sections of ePTFE–POC–Heparin grafts (5 mm in length) were pre-weighed and placed in 1.5 mL centrifuge tubes. As a control, non-modified ePTFE and POC-coated ePTFE samples were used. The anticoagulated whole blood samples were re-calcified with the addition of 10% (v/v) 0.1 M CaCl₂ and then 750 μ L of re-calcified blood were then immediately incubated with graft samples for 1 h at room temperature. The grafts and all clotted blood were carefully removed, briefly blotted on a paper towel and weighed. The bioactivity of POC–Heparin ePTFE coating was evaluated

after 14 days and 28 days of incubation *in vitro* at 37 °C in PBS. To evaluate the heparin bioactivity after adsorption with plasma proteins, samples were incubated with platelet poor plasma for 1 h at 37 °C prior to the whole blood clotting assay. Previous plasma protein studies have determined that a majority of protein becomes adsorbed to heparin-coated surfaces within this time frame [29]. To account for the variability between blood donor collections, each blood clotting experiment included a set of non-modified ePTFE control samples to normalize the whole blood clot mass for POC and POC–Heparin experimental samples.

2.6.2. Platelet adhesion—Whole blood was collected from adult volunteers into ACD anticoagulant, which has previously been reported to preserve platelet activity [13]. The blood was centrifuged at 250 g for 15 min to obtain platelet-rich plasma (PRP) supernatant. The PRP preparation and platelet suspension buffer (PSB) used for this experiment were described previously [30]. Samples of ePTFE were cut into disks using a cork borer to match the surface area of 96 multiwell plates and gently pinned down to remain in place. PRP, diluted 1:10 in PSB, was incubated at 37 °C for 60 min with prepared ePTFE samples and gently rinsed with warm PBS. The number of adherent platelets was determined by detecting the amount of lactate dehydrogenase (LDH) present after cell lysis as previously described [15]. Briefly, adherent platelets were lysed by incubation with 2% Triton-PSB buffer for 30 min at 37 °C. A colorimetric substrate for LDH (Roche Diagnostics Corporation, Indianapolis, IN) was added and incubated for 20 min at 37 °C. The reaction was stopped with the addition of 1N hydrochloric acid. The optical density was measured at 490 nm with a reference wavelength of 650 nm. A calibration curve was generated from a series of serial dilutions of a known platelet concentration and used to determine the number of adhered platelets. The morphology of adhered platelets was assessed via scanning electron microscopy (SEM). Briefly, adherent platelets were fixed using 2.5% glutaraldehyde in PBS for at least 2 h, dehydrated in a graded series of ethanol, and freeze-dried. The samples were then sputter-coated with a 7-nm layer of gold and observed using scanning electron microscopy (SEM 3400N, Electron Probe Instrumentation Center, Northwestern University).

2.7. Effect of immobilized heparin on vascular cells

Human umbilical vein endothelial cells (HUVEC) (Lonza, Baltimore, MD) (passages 3–6) were cultured in EGM-2 media. Human aortic smooth muscle cells (HASMC) (Lonza) (passages 3–5) were cultured in SmGM-2 media. BOECs were isolated and cultured as described earlier. All cells were cultured at 37 °C in a humidified incubator containing 5% CO₂. HUVECs and HASMCs (seeding density 1×10^4 cells/cm²) were seeded onto POC, POC–Heparin and TCP surfaces. Similarly, the seeding density for BOECs was 7.5×10^3 cells/cm². Cell culture media was changed every 3 days.

2.7.1. Cell proliferation—At predetermined time intervals, cells were lysed and quantified using a Pico- Green DNA assay (Molecular Probes, Carlsbad, CA) and compared against standards of known cell numbers

2.7.2. Cell viability—Cell viability of adherent cells was assessed using a live/dead cell viability assay kit (Invitrogen, Carlsbad, CA) after 4 days of culture. Following manufacturer's instructions, after incubation with the live/dead staining solution, the cells were gently rinsed in warm PBS and adherent cells were imaged for viability by fluorescence microscopy.

2.7.3. Cell phenotype—Cells were fixed with 4% paraformaldehyde and blocked with 10% normal goat serum. HUVECs and BOECs were probed with primary antibodies to EC-specific markers von Willebrand factor (vWF) (Dako Cytomation, Carpinteria, CA) and CD144 (VE-Cadherin) (R&D Systems, Minneapolis, MN). HASMCs were probed with α -actin smooth muscle (abCam, Cambridge, MA). Cells were counterstained with Hoechst and analyzed by fluorescence microscopy (Nikon TE2000U). POC and POC-Heparin surfaces have background fluorescence when stained with Hoechst; therefore, the background fluorescence was digitally removed for clarity.

2.7.4. Nitric oxide production—HUVECs and BOECs were cultured on TCP, POC and POC-Heparin surfaces for up to 4 days and then probed for production of nitric oxide using the fluorescent probe 5,6-diaminofluorescein diacetate (DAF-2 DA, Santa Cruz Biotechnology, Santa Cruz, CA) [31]. After cell detachment with trypsin-EDTA, cell suspensions were incubated with 5 μ M DAF-2 DA for 1 h at 37 °C. The fluorescence of DAF was excited at 488 nm and emitted fluorescence was measured at 530/40 nm using a BD LSR2 flow cytometer (BD Biosciences, San Jose, CA).

2.8. Statistical analysis

Numerical data are reported as mean $\pm$ standard deviation (SD). The statistical significance between two sets of data was calculated using a two-tail Student's *t*-test. One way and two way ANOVA tests were used to measure differences for experiments with multiple data sets with a *post hoc* Bonferroni test performed between groups with significant differences to correct for the multiple pairwise comparisons. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Heparin immobilization to POC

The covalent modification of thin POC films with diaminohexane and heparin was assessed by XPS for N1s and S1s spectra (Fig. 1). The presence of nitrogen from diaminohexane amine functional groups was detected for POC-diaminohexane in addition to POC-Heparin surfaces. As expected, no nitrogen or sulfur peaks were detected for unmodified POC surfaces and no sulfur peaks were detected for POC-DH surfaces. Surfaces conjugated with heparin showed peaks for sulfur.

3.2. Fabrication of the POC-Heparin ePTFE graft

Vascular grafts were prepared by coating ePTFE tubes with a coating of POC using a "spin shear" coating technique developed in our lab [13]. The POC coating was used to prepare the ePTFE for subsequent covalent immobilization of heparin via a DH linker molecule

(Fig. 2). The POC pre-polymer is cross-linked within the node and fibril structure of the ePTFE lumen at 80 °C. The POC and POC–Heparin coatings are capable of modifying the ePTFE without significantly altering the original node and fibril architecture (Fig. 3).

The presence of heparin on the luminal surface of modified ePTFE grafts was confirmed visually by a purple color change from the toluidine blue stain (Fig. 3D). The cationic toluidine blue dye can also bind to negatively charged carboxyl groups present in POC and showed significant background staining for POC-coated ePTFE. The purple color change was only seen on POC–Heparin ePTFE. No dye binding was observed for ePTFE control grafts. The toluidine blue dye was also used to quantify the surface density of POC–Heparin-coated ePTFE grafts and confirm the stability of the immobilized heparin after incubation under physiological conditions *in vitro* (Fig. 3E). There were no statistically significant changes in the heparin surface density over time ($p = 0.58$). The heparin surface density after incubation for 14 days and 28 days was 35.5 ± 11.1 and 37.4 ± 8.9 ng/mm², respectively, compared with 47.2 ± 14.6 ng/mm² for freshly prepared samples.

Static water-in-air contact angle measurements also confirmed the successful modification of the ePTFE luminal surface with a coating of POC and surface immobilized heparin. After a 5-min time period, the POC–Heparin ePTFE had a significantly reduced water-in-air contact angle compared with ePTFE and POC–ePTFE surfaces (Fig. 3F). After 15 min, ePTFE, POC–ePTFE and POC–Heparin ePTFE had water contact angles of $98.43 \pm 5.32^\circ$, $86.67 \pm 6.53^\circ$ and $20 \pm 18.17^\circ$, respectively.

3.3. Bioactivity and antithrombogenicity assessment of POC–Heparin ePTFE grafts

3.3.1. Re-calcified whole blood clotting—The bioactivity of POC–Heparin ePTFE grafts was assessed over a 28-day incubation *in vitro* at 37 °C in PBS. A re-calcified whole blood clotting assay demonstrated the potent anticoagulant activity of the POC–Heparin ePTFE grafts (Fig. 4). The POC–Heparin grafts had a dramatic effect upon whole blood clotting for all time points tested: Days 0, 14 and 28. Upon visual inspection, the POC–Heparin graft surface remained relatively clean compared with the POC-coated and bare ePTFE grafts (Fig. 4A). The POC–Heparin grafts had significantly less blood clot formation compared with ePTFE and POC–ePTFE grafts at all time points tested up to 28 days ($p < 0.05$). In addition, the POC–Heparin grafts had no significant change in anti-clotting activity over the same time period. In contrast, the POC-coated grafts were not significantly different from ePTFE controls at all time points tested. At the 28-day time point, the POC–Heparin and POC–ePTFE samples had blood clot formation that was $15.6 \pm 10.2\%$ and $70.6 \pm 24.4\%$ of ePTFE controls, respectively.

The POC–Heparin ePTFE samples were also evaluated for anticlotting activity after incubation in 100% platelet poor plasma for 1 h at 37 °C. The POC–Heparin samples pre-incubated in plasma had significantly less whole blood clot formation which was only $4.6 \pm 5.7\%$ compared with ePTFE control grafts ($p < 0.05$) (Fig. 5).

3.3.2. Platelet adhesion—In addition to whole blood clotting, the grafts were evaluated for platelet adhesion using platelet-rich plasma diluted in platelet suspension buffer (Fig. 6). Platelet-rich plasma contains clotting factors present in the plasma as well as proteins such

as fibrinogen, contained within the α -granules of platelets, which are capable promoting clot formation [32]. There were numerous adherent and spread platelets within a clot on the luminal surfaces of both POC-coated and ePTFE control grafts as visualized by SEM (Fig. 6A and B). The adherent platelets as seen by SEM are approximately 2.5 μm in diameter which corresponds to average platelet size in humans and the adhered and spread platelet morphology is similar to previously reported literature [33,34]. In comparison, there was a dramatic difference with the POC–Heparin grafts which remained relatively pristine with the ePTFE node and fibril architecture still clearly visible (Fig. 6C). An LDH assay quantified the number of adherent platelets on the different vascular graft surfaces. The number of adherent platelets on POC–Heparin ePTFE grafts was significantly less than POC and ePTFE grafts ($p < 0.05$). The number of platelets on POC and ePTFE grafts was $4.5 \times 10^7 \pm 3.5 \times 10^6$ and $4.8 \times 10^7 \pm 5.8 \times 10^6$ per cm^2 , respectively. In contrast, the number of adherent platelets to POC–Heparin grafts was only $1.5 \times 10^6 \pm 4.7 \times 10^5$ per cm^2 . The relatively small number of adherent platelets on the POC–Heparin surface as visualized by SEM and quantified by the LDH assay, demonstrates the ability for the POC–Heparin surfaces to strongly inhibit platelet adhesion when challenged with a relatively high concentration of platelets (platelet-rich plasma).

3.4. Effect of immobilized heparin on vascular cells

3.4.1. HUVEC and BOEC viability and proliferation on POC–Heparin—HUVECs and BOECs exhibited good attachment, spreading, and a high degree of viability on all surfaces tested (Fig. 7). In addition, HUVECs and BOECs stained positive for vWF and VE-Cadherin (Fig. 8). POC–Heparin supported cell proliferation for both endothelial cell types although there was some inhibition of HUVECs proliferation on POC–Heparin (Fig. 9). Specifically, the HUVEC surface density after 4 days on POC–Heparin was 1.25×10^5 cells/ cm^2 compared with 1.63×10^5 cells/ cm^2 and 1.50×10^5 cells/ cm^2 for TCP and POC surfaces, respectively. Although there were significantly fewer HUVECs on the POC–Heparin surfaces after 4 days, the HUVECs were capable of forming a confluent cell monolayer on the POC–Heparin surface after 7 days of culture (Fig. 9). Additionally, HUVECs maintained expression of endothelial cell markers and exhibited no signs of decreased cell viability on POC–Heparin. In contrast, BOECs proliferated at the same rate on all surfaces, although at a slower rate when compared to HUVECs. The BOEC surface density after 4 days on POC–Heparin was 1.12×10^4 cells/ cm^2 compared with 1.15×10^4 cells/ cm^2 and 1.27×10^4 cells/ cm^2 for TCP and POC surfaces, respectively. The number of BOECs on the different surfaces after 4 days of culture was not statistically significant.

3.4.2. HASMC viability and proliferation on POC–Heparin—HASMCs showed good adhesion and viability on all surfaces tested with minimal signs of dead/dying cells (Fig. 7). The HASMCs on POC and POC–Heparin also had a more elongated and less spread morphology compared with HASMCs cultured on TCP. Interestingly, HASMCs cultured on POC and POC–Heparin surfaces had greater α -actin expression, which is an indicator of a more physiological contractile phenotype (Fig. 10B). For HASMC proliferation, there were significantly fewer cells on both POC and POC–Heparin surfaces after 4 days of culture (Fig. 10A). The HASMC surface density after 4 days on POC–Heparin was 7.39×10^4 cells/ cm^2 compared with 1.32×10^5 cells/ cm^2 and 5.06×10^4

cells/cm² for TCP and POC surfaces, respectively. The proliferation data is inline with the α -actin staining, which demonstrates that POC and POC–Heparin surfaces promote a more contractile and less proliferative smooth muscle cell phenotype.

3.4.3. Nitric oxide production—HUVECs and BOECs cultured on POC and POC–Heparin surfaces produced similar levels of nitric oxide as cells cultured on TCP (Fig. 11). These results demonstrate the ability for POC–Heparin surfaces to support functional nitric oxide producing cells normally present in healthy endothelium.

4. Discussion

The quest for the ideal prosthetic vascular graft has generated a significant amount of research on the development of novel biomaterials and surface modification techniques to improve the clinical outcome of bypass surgeries. Of the various strategies investigated over the years, only endothelial cell-based and heparin-based approaches have shown significant promise in regards to patient outcome [35,36]. Nevertheless, more research is needed to overcome the challenges associated with the *in vitro* or *in vivo* endothelialization of prosthetic grafts to enable widespread clinical use.

With regard to heparin immobilization, non-covalent and covalent strategies have been reported with the latter more desirable to minimize the release of heparin into the systemic circulation [11,37]. Although a comprehensive review of all antithrombogenic strategies for cardiovascular biomaterials is beyond the scope of this current paper, Table 1 provides a summary of heparin-modification strategies and current technologies that are specific to the development of vascular graft biomaterials. A more detailed review of antithrombogenic coating technologies for vascular grafts has been prepared by Tatterton et al., and Kapadia et al. [38,39]. In recent years, there has been a growth of commercially available heparinmodified vascular graft materials such as the Propaten[®] graft (W.L. Gore & Associates, Inc.) and the Intergard[®] graft (Maquet Cardiovascular Inc.). However, the long-term performance and benefit of such commercially available technologies remains to be answered as the Intergard[®] graft has demonstrated no difference in performance after 5 years compared with human umbilical vein grafts and PTFE grafts [40,41]. Furthermore, the longest prospective and randomized clinical trial to date for the Propaten[®] graft is only for 1 year, but results from this study reveal a decrease in primary graft failure compared with PTFE as a bypass for lower limb ischemia [42].

The Carmeda Bioactive Surface Modification (CBAS[®]) technique is the basis for the Propaten[®] vascular graft manufactured by W.L. Gore, which is among the most widely used of commercially available heparin-bonded vascular grafts [7,43]. CBAS[®] is based on layer-by-layer deposition of oppositely charged polyelectrolytes, followed by covalent attachment of heparin, via its reducing end, to polyethyleneimine. The CBAS[®] technology requires toxic chemical reagents and numerous surface modification steps involving nitrous acid-treated heparin and cross-linking of polyethyleneimine layers with glutaraldehyde [7,44]. Previous work has shown that biomaterial surfaces prepared with polyethyleneimine by layer-by-layer deposition exhibit cytotoxicity and inhibit cell proliferation [45].

In this study, we developed a new and easily implemented approach to covalently link bioactive heparin to the lumen of ePTFE grafts using a thermally cross-linked POC elastomer and immobilization chemistry that targets the carboxyl groups on heparin. As endothelial and smooth muscle cells play an important role in intimal hyperplasia and blood vessel homeostasis, the effects of immobilized heparin on these cell types were also evaluated. The POC copolymer chain is composed of a large number of carboxyl and hydroxyl functional groups that are amenable for a variety of surface functionalization strategies for tailoring surface chemistry and/or immobilizing bioactive molecules or peptides. The copolymer can be readily coated onto the nodes and fibrils of ePTFE vascular grafts (Fig. 3) without significantly altering vascular wall thickness, mechanical properties, or ePTFE node/fibril microstructure [16].

The stability of surface bound molecules for improving vascular conduit blood contacting properties is crucial for long-term vascular graft performance and patency. For example, large amounts of eluted heparin can potentially become lethal due to complications associated with heparin-induced thrombocytopenia [46]. The heparin surface density on POC-Heparin ePTFE grafts ($\sim 36\text{--}46\text{ ng/mm}^2$) remained stable over a 28-day period *in vitro* under physiological conditions with no significant change in heparin surface density over time. In contrast, although heparinized stainless steel stents have similarly reported values, they experience considerable degradation (nearly 40%) after one month [33]. Other previous work with heparinization of polyurethanes has shown a maximum heparin surface density of approximately $11\text{--}23\text{ ng/mm}^2$ with stability evaluated after only 4 days [47–49].

It is important to evaluate the activity of immobilized heparin because heparin surface density is not necessarily proportional to antithrombogenic activity [50,51]. Aside from factors such as heparin molecular weight and purification methods which may affect heparin activity, the type of covalent modification may alter accessibility of the heparin's ATIII-binding site [50]. In this report, POC-Heparin-coated ePTFE grafts significantly inhibited whole blood clotting and maintained long-term bioactivity *in vitro* for up to one month. These results are promising because other studies have shown inconsistent immobilized heparin bioactivity after much shorter time scales (~ 5 days) [52]. In this regard, the use of spacer molecules such as acrylamide has been shown to improve the bioactivity of immobilized heparin [50]. In this work, the diamino hexane linker likely allows for improved bioactivity due to increased mobility of heparin's ATIII-binding site. Activation of ATIII and subsequent inhibition of pro-clotting factors involved in coagulation are key to reduced blood coagulation when blood comes in contact with surfaces displaying immobilized heparin [53]. The anticoagulant property of immobilized heparin in the grafts was also maintained after incubation with human plasma. This characteristic is important because clotting factors present in the plasma may adsorb to a blood contacting surface leading to activation of the coagulation cascade [54]. It is believed that surfaces with immobilized heparin are capable of inhibiting the process of serum protein adsorption, activation and denaturation involved in thrombus formation [29,55].

Furthermore, heparin is capable of inhibiting platelet adhesion and activation in the presence of ATIII, but may cause platelet aggregation under certain conditions depending on the molecular weight fraction and concentration [56]. POC-Heparin vascular grafts described

herein significantly inhibited platelet adhesion as verified by LDH activity and SEM imaging. Additionally, hydrophilic surfaces are associated with improving biocompatibility while inhibiting platelet adhesion and activation [57]. In this regard, the POC–Heparin coating dramatically improved the wettability of the ePTFE vascular graft surface. The covalently attached heparin molecule via the diaminohexane linker may create an ideal hydrophilic layer for further inhibiting platelet adhesion. Although POC has been previously shown to inhibit platelet adhesion, POC cross-linking and rinsing conditions can affect POC surface energy and charge density therefore affecting its interaction with platelets when in contact with blood [16]. Therefore, incorporating heparin into the POC to provide a more robust inhibition of platelet adhesion is warranted.

One of the reasons for the poor patency of small-caliber ePTFE grafts is due to intimal hyperplasia resulting from the migration and over proliferation of vascular smooth muscle cells [58]. Therefore, the POC–Heparin material was characterized for *in vitro* compatibility of HASMCs because of the involvement of smooth muscle cell pathology in cardiovascular disease and vascular graft failure. POC and POC–Heparin surfaces were capable of reducing HASMC proliferation and elevating expression of smooth muscle α -actin protein. These results are important because increased HASMC proliferation and reduction in contractile phenotype markers such as α -actin, are implicated in stenosis progression leading to graft failure [59]. Furthermore, heparin signaling and substrate compliance are known to alter HASMC proliferation and phenotype [60,61]. Our findings are noteworthy in that they are the first to show that poly(diols citrate) elastomers modified with heparin are capable of modulating the phenotype of vascular smooth muscle cells in possible combination with heparin signaling and polymer substrate compliance and warrant further investigation regarding the interactions between HASMCs and elastomeric poly(diols citrate) biomaterials.

Although previous studies have demonstrated good endothelial cell and BOEC compatibility with unmodified POC surfaces, the covalent modification with heparin and its resulting effects on cell behavior must be investigated [13,15]. POC–Heparin surfaces supported adhesion, spreading and proliferation of both BOECs and HUVECs. Although the presence of heparin seems to have had an effect on HUVEC proliferation, cells were viable and maintained an endothelial cell phenotype. Nitric oxide secretion is an important endothelial cell mediated process for maintaining a physiologically healthy endothelium and inhibiting thrombus formation. Endothelial function was further confirmed by verifying the production of NO. BOECs and HUVECs had comparable NO production when cultured on POC–Heparin.

There is a limited amount of information in the literature regarding the simultaneous characterization of vascular graft surfaces enhanced with antithrombogenic activity and the resulting influence on endothelialization and smooth muscle cell function. It is also well known that novel biomaterials used in vascular grafts may also adversely affect endothelial cell function [62]. Moreover, EPC seeding strategies for improving vascular graft thromboresistance have heavily relied on the incorporation of collagen, fibrin and fibronectin for improving cell compatibility [63–65]. However, these extracellular matrix and plasmaproteins also promote platelet adhesion and thrombus formation and a subconfluent or denuded endothelialized surface may provide nucleation sites for thrombus

formation. Therefore, the development of POC–Heparin as a multifunctional biomaterial is a significant step towards improving vascular graft performance since it is capable of inhibiting platelet adhesion, blood coagulation and vascular smooth muscle cell growth while simultaneously supporting endothelialization.

5. Conclusion

In this report we describe a new approach to impart heparin-mediated thromboresistance and vascular cell compatibility to vascular grafts. The POC–Heparin-coated vascular grafts remained bioactive and significantly inhibited whole blood clotting and platelet adhesion. POC–Heparin supported BOEC proliferation and expression of endothelial cell-specific phenotype markers and the production of nitric oxide. Furthermore, POC–Heparin modulated HASMC phenotype via elevated contractile protein expression and decreased cell proliferation rate. Our results support the feasibility of using BOECs and mature endothelial cell types for *ex vivo* or *in situ* endothelialization strategies. Due to the ease of synthesis and fabrication, the strategy described herein can be readily adopted to modify other types of devices such as stents, heart valve replacements devices and hemodialysis tubing.

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Fig. 1.
Detection of immobilized heparin by X-ray photoelectron spectroscopy. XPS spectra for N(1s) and S(1s) for POC, POC conjugated with DH (POC-DH), and POC-Heparin.

Fig. 2.
Schematic of the bioactive POC-Heparin ePTFE vascular graft.

Fig. 3. Characterization of POC-Heparin vascular grafts. SEM micrograph of the luminal surface of (A) unmodified ePTFE, (B) POC-coated ePTFE and (C) POC-Heparin-coated ePTFE. Arrows indicate areas with POC-coated fibrils (scale bars: 20 μ m). (D) POC-Heparin coating on ePTFE grafts was determined by toluidine blue staining showing a purple color change, *en face* preparations of graft segments show lumen side up. (E) Heparin surface density on freshly prepared (Day 0) POC-Heparin-coated ePTFE grafts and after 14 days and 28 days incubation *in vitro* at 37 °C in PBS, N.S. = “not significant”, $n = 6$, mean $\pm$ SD.

(F) Static water-in-air contact angle measurements for ePTFE, POC–ePTFE and POC–Heparincoated ePTFE grafts. * $p < 0.05$, significantly less than ePTFE, ** $p < 0.01$, significantly less than ePTFE and POC, *** $p < 0.001$ significantly less than ePTFE and POC, $n = 4$, mean $\pm$ SD. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

Fig. 4.

Bioactivity of POC-Heparin grafts over time. (A) Whole blood clot formation on ePTFE graft segments, POC, and POC-Heparin-coated ePTFE graft segments after 28 days incubation *in vitro* at 37 °C in PBS. Left image panels show graft lumen *en face* and right image panels show graft cross-sections. (B) Whole blood clot mass for POC and POC-Heparin-coated ePTFE as percent of ePTFE control surfaces, * $p < 0.05$, significantly less than ePTFE and POC. $N = 4$, mean $\pm$ SD. Note: ePTFE control samples ($N = 4$) were also

included for each blood clotting experiment (time points Day 0–Day 28) and used to normalize whole blood clot mass for POC and POC–Heparin samples.

Fig. 5.

POC-Heparin coating remains bioactive when exposed to human plasma. Whole blood clot mass for ePTFE and POC-Heparin grafts pre-incubated with platelet poor plasma as percent of ePTFE control surfaces, $*p < 0.05$ compared with ePTFE control and ePTFE +plasma samples, $n = 4$, mean $\pm$ SD.

Fig. 6. Effect of POC-Heparin on platelet adhesion. SEM micrographs of samples after incubation in platelet-rich plasma: (A) ePTFE, (B) POC-coated ePTFE and (C) POC-Heparin-coated ePTFE. (D) Platelet adhesion quantified by LDH, * $p < 0.05$ compared with ePTFE and POC samples, $n = 6$, mean $\pm$ SD. (A–C) Scale bars: 50 μ m.

Fig. 7. Cell viability of adherent cells on POC-Heparin. HASMCs, BOECs and HUVECs on TCP, POC and POC-Heparin surfaces after culturing for 4 days. Green: live cells, Red: dead cells. Scale bars: 100 μ m. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

Fig. 8.
 The effect POC-Heparin on endothelial cell phenotype. (A) Immunofluorescence staining for HUVECs and BOECs, Red: vWF, Green: VE-Cadherin, Blue: cell nuclei (scale bars: 100 μ m). [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

Fig. 9. HUVEC and BOEC cell growth on POC-Heparin. (A) Cell surface density for HUVECs and BOECs on TCP, POC and POC-Heparin surfaces. White bars = TCP, gray bars = POC, black bars = POC-Heparin surfaces for all panels. # $p < 0.01$ compared with POC, ** $p < 0.001$ compared with TCP. $n = 5$, mean $\pm$ SD. (B) Confluent monolayer of HUVECs after 7 days of culture on POC-Heparin (scale bar: 100 μ m).

Fig. 10.

The effect of POC-Heparin on smooth muscle cell growth and phenotype. (A) Cell surface density for HASMCs on TCP, POC and POC-Heparin surfaces. # $p < 0.01$ compared with POC, ** $p < 0.001$ compared with TCP. $n = 5$, mean $\pm$ SD. (B) Immunofluorescence staining for HASMCs, Green: α -actin, Blue: Cell nuclei (scale bars: 100 μ m). [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

Fig. 11.

Nitric oxide production in HUVEC and BOEC cultured on POC-Heparin. NO-positive cells were analyzed by flow cytometry using DAF-2 DA. HUVECs (A) and BOECs (B) cultured on TCP, POC and POC-Heparin surfaces. NO-positive cells were compared relative to “background” samples for which DAF-2 DA cell treatment was omitted.

Table 1
Current technologies and methods for developing heparin-modified vascular graft biomaterials.

Author/Company	Heparin incorporation technique/coating technology	Biomaterial	Longest performance time point measured	Outcome	Clinically available
Lord et al. [3]	Surface adsorption of perlecan heparan sulfate proteoglycan	ePTFE vascular grafts	6 weeks <i>in vivo</i> (ovine model)	↓ platelet adhesion <i>in vitro</i> (fresh samples), improved EC coverage and decreased platelet adhesion <i>in vivo</i> (qualitative analysis only at 6 weeks)	
Janaïro et al. [66]	EDC/NHS using diamino-PEG linker	Electrospun PLLA vascular graft	1 month <i>in vivo</i> (rat model)	Heparin-modified grafts had 86% patency compared with 43–57% for control grafts.	
Chuang and Masters [49]	PEI modified polyurethane, aldehyde activated heparin	Polyurethane films	5 days <i>in vitro</i>	↑ platelet adhesion <i>in vitro</i> (fresh samples), supported EC proliferation (5 days)	
W.L. Gore & Associates, Inc.	Carmeda Bioactive Surface Technology (CBAS®): layered PEI/dextran sulfate/glutaraldehyde, aldehyde activated heparin [44]	Propaten® PTFE vascular graft	Canine carotid artery interposition model; 3 months.	Canine model: ↑ graft patency versus ePTFE control grafts and no change in heparin activity [67].	Yes
Jotec GmbH	Flowline Bipore® Heparin: electrostatic bonding interactions/protein substrate	PTFE vascular graft	Randomized clinical trial: 1 year for bypass for lower limb ischemia	Clinical trial: ↓ risk of primary graft failure by 37% compared with PTFE [42].	European CE Mark Approval only
Maquet Cardiovascular	Bioline® coating: recombinant albumin and covalently attached heparin	Fusion Bioline® vascular graft, ePTFE and PET (Dacron®)	Clinical trial comparing femoropopliteal bypass (2004-present)	NA	European CE Mark Approval only
Intervascular Inc. (Acquired by Maquet in 2009)	Heparin-bonded collagen coating	Intergard® composed of PET (Dacron®) vascular grafts	FINEST Phase 3 clinical trial for peripheral artery disease (2011-ongoing)	NA	European CE Mark Approval only
			Prospective randomized clinical trial: 5 years for above-knee femoropopliteal bypass [40].	No difference in primary patency at 5 years compared with human umbilical vein grafts [40].	Yes
			Prospective randomized clinical trial: 5 years for above/below-knee femoropopliteal bypass [41].	Significantly improved patency at 3 years, but no difference at 5 years compared with PTFE grafts [41].	
Perouse Medical	Heparin bioactive luminal coating	PM® Flow Plus Heparin vascular graft, ePTFE	NA	NA	European CE Mark Approval only

NA = not available; PEI = polyethylenimine.

Note: Other clinically available heparin-bonded biomaterials include: Duraflon® II (Baxter International Inc.), Photolink® (SurModics Inc.) and Astute® Advanced Heparin Coating (BioInteractions Ltd.) marketed as Trillium Biosurface® (Medtronic Inc.) as hemocompatible coating technologies, but are not currently used for vascular grafts.