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***In vitro* and *ex vivo* hemocompatibility of off-the-shelf modified poly(vinyl alcohol) vascular grafts**

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Abstract

Synthetic small diameter vascular grafts with mechanical properties of native arteries, resistance to thrombosis and capacity to stimulate *in situ* endothelialization are an unmet clinical need.

Poly(vinyl alcohol) hydrogel (PVA) is an excellent candidate as a vascular graft due to its tunable mechanical properties. However, the hydrophilicity and bio-inertness of PVA prevents endothelialization *in vivo*. We hypothesize that the modification of PVA with biomolecules and topographies creates a hemocompatible environment that also enhances bioactivity. PVA modified with fibronectin, RGDS peptide, cyclicRGD (cRGD) peptide, or heparin provided cell-adhesion motifs, which were confirmed by detection of nitrogen through X-ray photoelectron spectroscopy. Protein- and peptide- modified surfaces showed a slight increase in human vascular endothelial cell proliferation over unmodified PVA. With the exception of fibronectin modification, modified surfaces showed *in vitro* hemocompatibility comparable with unmodified PVA. To further improve bioactivity, cRGD-PVA was combined with gratings and microlens topographies. Combined modifications of 2 μ m gratings or convex topography and cRGD significantly improved human vascular endothelial cell viability on PVA. *In vitro* hemocompatibility testing showed that topography on cRGD-PVA did not significantly trigger an increase of platelet adhesion or activation compared with unpatterned PVA. Using the more physiologically relevant *ex vivo* hemocompatibility testing, all PVA grafts tested showed similar platelet adhesion to ePTFE and significantly lower platelet accumulation compared to collagen-coated ePTFE grafts. The

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biochemical and topographical modification of PVA demonstrates excellent hemocompatibility with enhanced bioactivity of PVA, thus highlighting its potential as a vascular graft.

Graphical Abstract

Keywords

surface modification; luminal patterning; hemocompatibility; biomolecules; endothelialization

1. Introduction

Global demand for synthetic vascular grafts is increasing due to the prevalence and increased risk of occlusive cardiovascular disease. In the US alone, the market for vascular grafts is estimated to grow at 10% per year, with valuation at \$1.6 billion in 2005 [1]. Clinically utilized synthetic vascular grafts made from expanded polytetrafluoroethylene (ePTFE) and polyethylene terephthalate (Dacron) are bioinert, biocompatible and have shown long-term patency in diameters above 6 mm. However, when used in vessels with less than 6 mm diameter the patency rates are disappointing. After 5 years, ePTFE and heparin-bonded Dacron grafts achieved maximum patency of only 35% and 46%, respectively [2]. Stenosis and thrombosis of small diameter grafts are caused by the compliance mismatch between the synthetic graft and natural artery [3,4] and the lack of endothelialization of the graft [5].

One potential compliant biomaterial for vascular graft applications is poly(vinyl alcohol) (PVA), a synthetic, water-soluble polymer commonly used for biomedical applications [6–11]. Crosslinked PVA has high tensile strength and high water content, easily mimicking soft tissues. The ability to control crosslinking enables tuning of physical and mechanical properties without compromising tensile strength. Initial blood-contacting device studies that have used PVA were promising as demonstrated by its low platelet adhesion and long thrombin generation time [12]. Vascular grafts made from PVA showed 100% patency in 7 day implants into a rat abdominal aorta model [13]. Despite its excellent physical and mechanical properties, PVA is hydrophilic and biologically inert. Both *in vitro* and *in vivo* studies have failed to show any significant endothelial cell attachment on PVA surfaces [12,13], which would be necessary to induce *in situ* endothelialization. Thus, there is a critical need to perform surface modification to improve PVA bioactivity while ensuring hemocompatibility for vascular graft application.

A widely recognized method of improving bioactivity is to mimic the natural cellular niche [14]. Mimicking the topographically and biochemically complex environment of the

extracellular matrix (ECM) on synthetic substrates such as PVA may help direct specific biological actions such as improving cell adhesion and decreasing platelet activation. For example, mimicking the fibrillar ECM structure in the form of micron-sized gratings on synthetic surfaces has been shown to increase migration and retention under shear stress [15], improve proliferation [16] and retain the atheroprotective phenotype [17] of endothelial cells. Additionally, functionalization of synthetic surfaces with ECM proteins, such as fibronectin [18], vitronectin [19] and laminin [20] were shown to improve endothelial cell attachment. Anucleate platelets are also sensitive to both biochemical and biophysical cues [21,22], showing changes in morphology and activation. Despite the known benefits of combining biochemical and topographical cues, they have yet to be applied together to improve PVA for vascular graft applications.

We hypothesize that the introduction of a specific combination of biochemical and topographical cues onto PVA will improve its bioactivity without compromising hemocompatibility. In this study, we modified PVA surfaces with protein- (fibronectin), peptide- (RGDS and cRGD) and polysaccharide-(heparin) based biochemical cues. These modifications were tested for endothelial cell viability and proliferation, and *in vitro* blood compatibility. cRGD-PVA and PVA were then patterned with isotropic or anisotropic topographies and screened for endothelial cell adhesion and *in vitro* blood compatibility. The most favorable combination of biochemical and topographical modifications of PVA grafts were examined for hemocompatibility in a well-established *ex vivo* baboon shunt assay. These studies demonstrate the potential of PVA as a blood-contacting device and the potential to improve its bioactive properties using physical and biochemical surface modifications.

2. Materials and Methods

2.1 Preparation of PVA for crosslinking

PVA was crosslinked as previously described [23]. In brief, 10% aqueous solution of PVA (Sigma-Aldrich, 85–124 kDa, 87–89% hydrolyzed) was cross-linked with 15% (w/v) sodium trimetaphosphate (Sigma Aldrich) and 30% (w/v) NaOH. The crosslinking PVA solution was immediately casted into two-dimensional PVA films or three-dimensional tubular PVA grafts and was crosslinked at 18°C and 75–80% humidity for 5 days.

2.2 Biochemical modification of PVA

To biochemically modify PVA, the crosslinking PVA solution was mixed with bovine fibronectin solution (Biological Industries; 28.57 µg per g PVA), heparin (Leo Pharma; 142.86 U or 0.286 mg per g PVA), RGDS peptide (Sigma- Aldrich; 28.57 µg per g PVA) or cyclic RGD peptide [19] (cRGD, CRRGDWLC, Genscript Peptide Synthesis; 28.57 µg per g PVA). The amount of immobilized biomolecule and hydroxide content added to each gram of PVA was calculated (Table 1). PVA casting was carried out as described above in section 2.1.

2.3 Topographical modification of PVA films

Patterned molds of polydimethylsiloxane (PDMS) with specified topographies were fabricated from silicon molds using standard methods of soft lithography. Four topographies were studied and are summarized in Table 2: 1. Gratings with groove width, ridge widths and depth of $2\text{ }\mu\text{m} \times 2\text{ }\mu\text{m} \times 2\text{ }\mu\text{m}$ [2 μm gratings]; 2. Gratings with groove width, ridge widths and depth of $10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$ [10 μm gratings]; 3. Concave microlens structures with 1.8 μm diameter, 2 μm pitch and 0.7 μm sag [Concave]; and 4. Convex microlens structures with 1.8 μm diameter, 2 μm pitch and 0.7 μm sag [Convex]. PDMS cast on a plain tissue culture dish was used as an unpatterned template [Unpatterned]. Patterned PVA films are referred by its topography name. PVA films modified with cRGD are labelled as “topography+cRGD” or “Unpatterned+cRGD”. PVA films with neither pattern nor biochemical modification is referred to as “Unmodified” or “Unpatterned” control. The PDMS molds (2 cm $\times$ 1 cm) were used as templates for patterning tissue culture polystyrene (TCPS) through heat embossing. Next, 3.5 g of crosslinking PVA solution (section 2.1) or cRGD-PVA solution (section 2.2) was cast on patterned TCPS and allowed to crosslink. Patterned PVA films were immersed in sterile phosphate buffered saline (PBS) before demolding.

2.4 Surface characterization of PVA films

The surface chemical composition of PVA films was analyzed using X-ray photoelectron spectroscopy (XPS; Kratos Analytical) with spot size of $700\text{ }\mu\text{m} \times 300\text{ }\mu\text{m}$ and depth of 5 nm. A standard monochromated aluminum X-ray source (1486.7 eV) at 75 W was used to determine the survey and high-resolution spectra. Photoelectron detection was performed at 90° with respect to the surface plane. cRGD density on the surface of films were estimated by calculating molar ratio of PVA and cRGD detected on the surface, as indicated by the N/C ratio. PVA was taken to have an average molecular weight of 105 kDa and with average of 4776 carbon atoms per molecule.

The static water contact angle measurement was performed on dried PVA films (n=3) using VCA Optima (AST Products, Inc). One μl of DDI water was deposited on the surface of the PVA films and pictures were taken after the water droplet stabilized. Water contact angle on PVA films with gratings topography were examined along the gratings axis. The contact angle was measured with VCA AutoFAST software (AST Products, Inc).

2.5 Endothelial cell culture on PVA films

PVA films (1 cm $\times$ 1 cm) were placed into a 24-well plate and weighed down by Silastic tubing (Dow Corning) to yield an available PVA area of 0.6 cm^2 . PVA films were sterilized with ultraviolet light for 20 min and immersed in an aqueous solution of 20% penicillin-streptomycin and 2% amphotericin-B (Sigma-Aldrich) in sterile water. Immediately prior to use, PVA films were extensively washed in sterile water to eliminate any residual antibiotics and antimycotics. 500 μl of fetal bovine serum (FBS) was added to each PVA film. PVA films were centrifuged at 1000 rpm for 30 min and incubated overnight at 4°C prior to cell seeding.

Human umbilical vein endothelial cells (HUVECs, Lonza, P4 to P5) were grown in EGM-2MV growth medium (Lonza) at standard cell culture conditions of 37 °C and 5% CO₂. HUVECs were washed with HEPES buffered saline solution and trypsinized using 0.05% trypsin to harvest cells. A total of 200,000 cells cm⁻² in 500 µl of EGM-2MV medium was added to each PVA film. Seeded PVA films were incubated in standard culture conditions for 24 hrs.

2.6 HUVEC viability, adhesion, and proliferation

Cell viability was assessed at 24 hr after seeding using the Live/Dead assay kit (Life Technologies), used according to manufacturer's instructions. Viability was expressed as a ratio of signal from Calcein-AM (live dye) and Ethidium homodimer (dead dye). Cell adhesion was studied by fixing cells on PVA films after 24 hr. Cells were stained for fluorescent detection of actin and nucleus using Alexa Fluor-conjugated phalloidin (Life Technologies) and DAPI, respectively. Images of endothelial cell adhesion were taken using Leica SP5 confocal microscope. Click-iT EdU assay kit (Life Technologies) was used to quantify the percentage of proliferating cells. At 16 hrs post-seeding, ethidium homodimer (EdU; thymidine analog) was added to each PVA sample seeded with HUVECs. After 8 hrs, PVA samples were fixed and stained for EdU uptake, according to the Click-iT EdU assay kit (Life Technologies). Total HUVEC count was obtained by staining cells with nuclear stain DAPI (Life Technologies) and counting the number of nuclei using a fluorescence microscope (Leica). Proliferation was calculated as percentage of EdU positive cells compared to total cell number.

2.7 *In vitro* blood compatibility assays

2.7.1 Incubation with platelet rich plasma—*In vitro* blood compatibility assay was performed according to Yim *et al.* [24]. Blood samples were collected from healthy, male, New Zealand White rabbits (3.5–4.0 kg) in polypropylene tubes and primed with heparin (5U per ml blood). Blood was centrifuged at 100 g for 15 mins at 22 °C to collect platelet rich plasma (PRP). PRP (150 µl) was then added to each PVA film held down in a 24-well plate with Silastic tubing to ensure no contact of PRP on well plate. Glass coverslips incubated overnight with 0.1 mg bovine collagen I (Gibco) were included as a positive control. Rest samples of 50 µl PRP in the absence of PVA were included. Samples were incubated on an orbital shaker for 1 hr at 37 °C and 60 rpm. Non-adhered PRP solution was subsequently collected for flow cytometry analysis (section 2.7.2), while PVA films were kept for either scanning electron microscopy (SEM) analysis (section 2.7.3) or lactate dehydrogenase (LDH) assay (section 2.7.4).

2.7.2 Flow cytometric analysis of platelet activation on PVA films—PRP was collected from PVA films and placed in 1.5 ml polypropylene tubes. PRP was diluted with 200 µl of HEPES-Tyrodes buffer (HTB; 137 mM sodium chloride, 2.7 mM potassium chloride, 16 mM sodium bicarbonate, 5 mM magnesium chloride, 3.5 mM HEPES, 1% glucose, 2% bovine serum albumin, pH 7.4) and incubated with antibodies against GPIIb/IIIa (Abbiotec). Platelets were then washed with HTB and centrifuged at 21000 rpm for 5 mins at 4°C. Platelets were incubated with Alexa Fluor secondary antibodies (Life Technologies). Samples were fixed with 2% paraformaldehyde in HTB before analysis of

scatter characteristics and expression of markers using BD LSR Fortessa. Flow cytometry data was analyzed using FlowJo 4.0. Microparticle formation was calculated as percentage of GPIIb/IIIa-positive microparticles over total platelet events [25].

2.7.3 SEM analysis of platelet morphology on PVA films—PVA films were washed in PBS and fixed using 2.5% glutaraldehyde at 4 °C for 2 hrs. The films were dehydrated using a series of increasing ethanol concentration solutions. Dried PVA samples were coated with 10 nm thick platinum (JEOL-JFC 1600 auto-fine coater). Topographical features of PVA were visualized using JEOL-JSM 6010LV scanning electron microscope at high vacuum and 10 kV.

2.7.4 Total platelet attachment to PVA films—LDH assay was used to characterize the amount of adherent platelets on the PVA films. PVA films were washed in 1X PBS then placed into a new 24-well plate. Platelets adhered onto PVA films were lysed with 1% Triton X-100 in 1X PBS at 37 °C for 1 hr at 100 rpm. The lysate was spun down at $100 \times g$ and moved to a different polypropylene tube. Lysates were stored at -20 °C until analysis using LDH assay kit (Roche) according to manufacturer's instructions. LDH content was given as absorbance, which correlates with platelet adhesion as described previously [26]. The assay was performed in quadruplicate for each group.

2.8 Fabrication of patterned grafts with and without cRGD modification

Patterned PDMS was used to construct PVA grafts with luminal topography. A thin film of PDMS elastomeric substrate was spin-coated onto patterned PDMS templates at 500 rpm for 1.5 min. The pliable PDMS film was cut to a width of 12 mm and a length of 6 cm. The PDMS film was then wrapped around a uniform cylindrical mold (outer diameter 3.75 mm) with the patterned surface facing outwards and aligned along the longitudinal axis of the mold. To improve hydrophilicity and wettability; the assembled mold was plasma cleaned four times for 15 s at low power (7.16 W) with 90° turns of the mold each time. The mold was immediately immersed in unmodified (section 2.1) or cRGD-modified (section 2.2) crosslinking PVA solution and sonicated for 1 hour at 57 kHz and 25°C. Next, 5 additional layers of crosslinking PVA or cRGD+PVA solutions were added through dip-casting, with a 15 min drying interval in between dips. An additional 7 layers of crosslinking PVA solution was added to all grafts. All PVA grafts were allowed to crosslink for 3 days before swelling in PBS and removal from the tubular mold.

2.9 Preparation of grafts for ex vivo study

Hydrated PVA grafts (7 cm in length) were connected to silicone tubing (Technical Products, Inc.) using rigid ePTFE tubing (Small Parts, Inc.) as inner cuffs. Parafilm (Bemis) was wrapped around the cuffs to secure the graft connection. Additional rigid ePTFE tubing was connected externally around the entire PVA graft with Parafilm to prevent device kinking or movement. ePTFE vascular grafts (W.L. Gore) were constructed as described previously to ensure a uniform transition between the graft and the silicone tubing [27] and used as a clinical, negative control. Similarly, the ePTFE grafts were coated with 1 mg ml⁻¹ equine collagen type I (Chrono-log Corp.) for use as a positive control. Segments of ePTFE were permeabilized with ethanol, rinsed with water, and exposed to the collagen solution.

Solutions were pressurized through the graft pores to ensure full coating. The collagen dried overnight, and then the samples were wrapped with Parafilm to prevent leaking of the permeabilized graft. Samples were exposed to the collagen solution again and then segments, 2 cm in length, were attached to the silicone shunt tubing.

2.10 *Ex vivo* blood compatibility study

All animals were housed and cared for by Oregon National Primate Research Center (ONPRC) staff according to the “Guide to the Care and Use of Laboratory Animals” prepared by the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (International Standard Book, Number 0-309-05377-3, 1996) and as approved by the ONPRC Institutional Animal Care and Use Committee.

Chronic baboon femoral arteriovenous shunts were performed as described previously [27]. In brief, autologous platelets were labeled with ¹¹¹-Indium prior to device testing and reinfused into the animal. Similarly, homologous fibrinogen was labeled with ¹²⁵-Iodine and injected into the blood. The femoral artery and vein access ports were extended with silicone tubing, which connected to the inlet and outlet sides of the device. The grafts were exposed to whole blood without anticoagulant or anti-platelet interventions for 60 min. ¹¹¹-Indium was quantified every 5 min at a 2 cm central region of the graft on a gamma scintillation camera (GE Model 400T Maxi-Camera, Fairfield, CT) to measure platelet accumulation. Blood flow was measured with a flow probe (Transonic Systems, Ithaca, NY) and controlled at 100 ml min⁻¹ with a clamp located distally from the device. After blood exposure, samples (n = 4) were thoroughly rinsed and fixed with 10% formalin in PBS. Central sections of 4 cm were retained for fibrinogen quantification. After allowing for the full decay of ¹¹¹-Indium (approximately 30 days), ¹²⁵-Iodine was quantified by a 1480 Wizard Gamma Counter (PerkinElmer, Waltham, MA).

2.11 Statistical analysis

All data points are shown as mean ± standard deviation. One-way or two-way ANOVA with Tukey’s *post-hoc* test was performed on all parametric data sets. Kruskal-Wallis test with Dunn’s *post-test* was performed on the non-parametric data set on LDH absorbance. Differences were taken to be statistically significant at $p < 0.05$. *Ex vivo* platelet data were analyzed with a repeated measures 1-way ANOVA with time as the within-subject factor and surface treatment as the between-subjects factor. To determine outliers, the fibrin data was analyzed using a boxplot (IBM SPSS Statistics 22) displaying the median, quartiles, and outlier values. The outliers, which were defined as samples between 1.5 and 3 times the interquartile range from the end of the box, were removed from the ANOVA analysis. For all the data presented in this manuscript in figures or tables, any two data points lacking statistically significant differences are assigned the same letter, regardless of other letters assigned to each data point.

3. Results

3.1 Biochemical modification alters surface properties of PVA films

PVA films were modified with fibronectin, cRGD peptide, RGDS peptide and heparin. The elemental composition of the PVA surface changed after the biochemical modifications, as detected by XPS (Table 3). Expectedly, Unmodified films were primarily comprised of carbon and oxygen species. Heparin films showed lower carbon and higher oxygen content, cRGD and Fibronectin films showed comparable levels with Unmodified films. The O/C ratio for all films was similar except for Heparin, which had a higher ratio. In addition, high-resolution spectra of C1s for modified films showed the retention of the characteristic CH₂ and C-OH bands for Unmodified PVA (data not shown). Nitrogen species contributed by biomolecules were detected on all modified films, and the N/C ratio was comparable between all modified films. Ultrastructure analysis using SEM showed that cRGD, fibronectin, RGDS surfaces were smooth and comparable with Unmodified films, while Heparin films contained micron-sized pits on the surface (Figure S1). The distribution of biomolecules in modified PVA was uniform (Figure S2).

The wettability of the modified films was assessed through water contact angle measurements (Table 3). Biochemical modification resulted in significantly increased water contact angle and thus decreased wettability compared to Unmodified films.

3.2 Biochemical modification of PVA films affects HUVEC proliferation

The biological activity of the modified PVA surfaces was studied by assessing HUVEC viability, adhesion, and proliferation (Figure 1). After 24 hrs, the viability of HUVECs on the Unmodified and modified films was unchanged. However, HUVEC viability on Fibronectin, RGDS and cRGD films showed an increasing trend toward improved viability compared with Unmodified films (Figure 1A). In contrast, HUVECs grown on Heparin and Fibronectin films showed marginal changes in viability. The adhesion study on the biochemically modified PVA films showed a significant increase in HUVEC number on Fibronectin, RGDS and cRGD films compared with the cell number on Heparin films (Figure 1B). A similar trend was observed, where peptide and protein modifications trended to increase HUVEC proliferation over 6 hours while heparin modification negatively modulated HUVEC proliferation when compared with Unmodified films (Figure 1C). Significantly more HUVECs proliferated on Fibronectin, RGDS, and cRGD films compared to HUVECs on Heparin films.

3.3 cRGD modification of PVA films does not alter *in vitro* hemocompatibility

Multiple methods for testing biomaterial hemocompatibility were used to quantify both adherent platelet and activated platelet populations after the platelet-material contact event. Examining both platelet populations enabled a clearer picture of the thrombotic potential of the modified PVA films. The LDH assay, as reported by Wu *et al.* [26], was used to assess the extent of platelet adhesion. SEM imaging was used to assess platelet morphology, which is indicative of the activation status. Detecting microparticles (pro-coagulant vesicles) released from platelets using flow cytometry is another easy, sensitive and well-established method to examine platelet activation as a marker of hemocompatibility [24, 28, 29]. The *in*

vitro blood compatibility assays were performed as a preliminary test to determine the hemocompatibility of modified films (Figure 2). Using the LDH assay, absorbance was not significantly altered on heparin, fibronectin, RGDS, and cRGD compared with Unmodified films (Figure 2A). Fibronectin films showed the highest absorbance among all the samples while cRGD films had very similar LDH absorbance to Unmodified films. Collagen-coated glass positive control exhibited the highest absorbance in the LDH assay.

The amount of microparticles released trended to be highest on platelets after contact with fibronectin films, which had a high degree of variability (Figure 2B). Yet microparticles released from platelets that were in contact with all the modified films did not show any significant change compared with Unmodified films. SEM images confirmed extensive platelet aggregation on fibronectin films, similar to the collagen-coated glass positive control (Figure 2C). There were also some platelet aggregation on the Heparin and RGDS, albeit to a lesser extent than Fibronectin films. Meanwhile, SEM images of cRGD films showed individual platelets attached without extensive dendritic extensions, indicating a less activated platelet phenotype.

Our results indicated that fibronectin modification was not suitable for vascular application of PVA due to induction of platelet activation. Likewise, heparin was deemed unsuitable due to the significantly low endothelial cell adhesion and proliferation on Heparin films. Thus, we chose cRGD modification for further testing because of the hemocompatibility and improvement in HUVEC proliferation shown on cRGD films.

3.4 Combination of cRGD and topography on PVA films

Topographical cues were then added to the Unmodified and cRGD films (Table 2). Both cRGD and Unmodified films were successfully patterned with isotropic and anisotropic topographies (Figure S3). Wettability of the patterned films was studied using water contact angle measurements (Table 4). Compared with Unpatterned films, 2 μ m gratings and 2 μ m gratings+cRGD films had a significantly increased contact angle. Additionally, 10 μ m gratings and 10 μ m gratings+cRGD showed a significant decrease in contact angle compared to Unpatterned and Unpatterned+cRGD films. Convex showed a significant decrease in contact angle while its combination with cRGD (Convex+cRGD) significantly increased contact angle when compared with Unpatterned+cRGD films. The contact angles of Concave and the combination with cRGD (Concave+cRGD) did not differ from the contact angle of the Unpatterned but was significantly lower than Unpatterned+cRGD films. Contact angles of 2 μ m gratings and Convex films significantly increased with the addition of cRGD. The contact angles of Concave and 10 μ m gratings were not significantly affected when modified with cRGD.

3.5 Topographical modification of cRGD films modulates HUVEC adhesion

Endothelial cell viability and HUVEC adhesion was analyzed on the patterned PVA films with and without cRGD (Figure 3). After 24 hrs of culture, HUVECs showed significantly higher viability on 2 μ m gratings, 2 μ m gratings+cRGD, 10 μ m gratings+cRGD, Convex, Convex+cRGD and Concave+cRGD compared with the Unpatterned (without cRGD) control (Figure 3A). The highest number of HUVECs adhered on 2 μ m gratings+cRGD,

which was significantly higher than the number of adhered HUVECs on Unpatterned (without cRGD) control (Figure 3B). HUVECs grown on 2 μ m gratings+cRGD and 10 μ m gratings+cRGD films aligned along the gratings axis compared with HUVEC on Unpatterned films (Figure 3C). Endothelial cells grown on films with microlens topography predominantly had circumferentially arranged actin fibers. There were no substantial differences in the number and morphology of adherent HUVECs on the remaining surfaces when compared with Unpatterned or Unpatterned+cRGD films.

3.6 cRGD films with topography retains hemocompatibility

The *in vitro* blood compatibility of all patterned films showed a general increase in LDH absorbance (Figure 4A) and microparticle release (Figure 4B) when compared to Unpatterned and Unpatterned+cRGD films. It was notable that while 2 μ m gratings and 10 μ m gratings films only showed small differences, all Convex and Concave films showed higher absorbance when compared to Unpatterned and Unpatterned+cRGD films (Figure 4A). Convex films had significant increase in LDH absorbance compared with Unpatterned films, but incorporation of cRGD resulted in similar LDH absorbance levels between Convex+cRGD and Unpatterned films. Modification with cRGD also significantly decreased microparticle release on 2 μ m gratings while it significantly increased on Convex films (Figure 4B). The cRGD modification appeared to have no substantial effect on microparticle release on 10 μ m gratings or Concave films. The SEM image of films after platelet adhesion indicated a general improvement in the morphology of platelets on all patterned cRGD films when compared with its unmodified patterned counterparts (Figure 4C), suggesting a decrease in platelet activation.

In summary, 2 μ m gratings+cRGD showed promising endothelial cell viability, adhesion and *in vitro* hemocompatibility. Thus, PVA films with 2 μ m gratings were chosen as the anisotropic pattern for further examination. Modified and unmodified Convex films showed statistically significant performance in endothelial cell viability and was hence chosen as the isotropic pattern for *ex vivo* shunt assay. Convex films also demonstrated a slightly positive effect on proliferation of HUVECs (Figure S4).

3.7 *Ex vivo* hemocompatibility assay shows retention of hemocompatibility for unmodified and cRGD grafts with patterns

PVA grafts with and without cRGD modification on no topography, 2 μ m gratings, or Convex microlens were fabricated and tested in a baboon *ex vivo* shunt assay. Platelet accumulation per unit length in 5 minute increments was obtained (Figure 5). The collagen-coated ePTFE positive control (collagen) had significantly more platelet accumulation than any other group. The unmodified, ePTFE clinical control (ePTFE) was statistically the same as the PVA grafts but had higher initial platelet adhesion with a different accumulation trend, with a maximum value of $0.52 \pm 0.26 \times 10^9$ platelets cm^{-1} at 45 mins and decreasing to $0.41 \pm 0.15 \times 10^9$ platelets cm^{-1} at 60 mins. The platelet adhesion on all of the PVA grafts was low with the average less than 0.41×10^9 platelets cm^{-1} throughout the 60 minute experiment.

Fibrin incorporation data per unit length are illustrated in Figure 5b. The collagen grafts had significantly more fibrin accumulation than the Unpatterned grafts without modification. Unmodified and cRGD-modified grafts with 2µm gratings and convex structures had significantly lower fibrin accumulation than collagen grafts. In general, the low fibrin incorporation was maintained in the PVA grafts with both the topographical and the biochemical treatments.

4. Discussion

Synthetic small diameter vascular grafts have a higher rate of failure due to the lack of mechanical compliance and endothelialization. One of the biomaterial solutions is to use PVA, which is biocompatible and hemocompatible with tunable mechanical properties to match native arteries. While it has been shown that PVA grafts have good short-term patency in a rat aorta implant, endothelialization of the luminal surface was not observed [13]. Biochemical and biophysical modifications, which have the potential to stimulate *in situ* endothelialization of PVA, also increase the risk of poor hemocompatibility. In this study, we showed that modification of the PVA surface with biochemical and topographical cues can be used to enhance endothelial cell adhesion without compromising hemocompatibility.

The inherent hydrophilicity of PVA prevents serum protein adhesion and deters cell adhesion [18,30,31]. To overcome this issue, biomolecules were crosslinked with PVA. We postulated that biomolecules would be tethered by crosslinking with the activated hydroxyl groups on PVA. According to Lack *et al.* [32], crosslinking of PVA with STMP proceeds first through the reaction of the pendant hydroxyl group on PVA with NaOH, forming the alcoholate group. The alcoholate group or NaOH in the solution then opens the STMP ring, resulting in polymer-grafted sodium tripolyphosphate (STPP) or simple STPP, respectively. Reaction of another alcoholate group with grafted STPP forms a monophosphate linkage to connect PVA moieties. The same activation process is likely to transpire on the multiple hydroxyl side chains of the biomolecules, resulting in its crosslinking to PVA. Thus, the incorporation of bioactive molecules in PVA was performed by directly mixing of these biomolecules with PVA solution and crosslinking in aqueous and ambient conditions.

The successful modification of the PVA surface with biomolecules was evidenced by the presence of nitrogen, as contributed by the biomolecule modifiers. In contrast, we and another group [12] have shown that Unmodified films only contains oxygen and carbon. The change in surface chemistry on the modified films was also observed with a significant increase in water contact angle. Despite these indications, there was a lack of any significant improvement in HUVEC viability and proliferation on the modified PVA surfaces. Crosslinking of the biomolecules to the PVA backbone may constrain the conformation of the biomolecules, leading to the lack of cell access to important moieties that aid cell adhesion and proliferation. For instance, the large molecule heparin contains a high density of hydroxyl groups that can be crosslinked to PVA, including sites that may improve cell adhesion by enhancing protein adsorption [33]. The significant decrease in cell adhesion observed on the Heparin films suggests that the crosslinking process may have prevented the relevant active sites from appearing on the surface. Conversely, fibronectin contains

thrombogenic and cell attachment moieties [34], both of which may have stimulated the HUVEC adhesion and platelet activation observed on Fibronectin surfaces. The limited active moieties and crosslinking sites on small RGDS and cRGD peptides may create attachment sites that are accessible to cells. This was manifested in the hemocompatibility of cRGD surfaces, similar to what has been reported previously [19].

We also observed that endothelial cells increased viability on cRGD films compared with Unmodified films. Using the N/C ratio from XPS data, we estimate that cRGD films have a density of 2.5×10^{11} cRGD ligands mm^{-2} on the surface. We obtained higher ligand density than what is needed for endothelial cell adhesion (6×10^6 linear RGD ligands mm^{-2}) [35] and achieved a level similar to the requirement for endothelial cell proliferation (3×10^{11} linear RGD ligand mm^{-2}) [36]. The difference between the linear RGD oligopeptide used by Hersel *et al.* [35] or Sagnella *et al.* [36], and the smaller cRGD and RGDS peptides used in this study may have contributed to the lack of significant change in endothelial cell adhesion, viability, and proliferation on cRGD films. Mobility of the polymeric chains that tether cRGD and RGDS peptides used for PVA modification may also prevent ligand clustering in the hydrogel, thus reducing integrin recruitment and activation on the endothelial cell surface. In contrast, both of the previous studies [35, 36] both used glass substrates immobilized with linear RGD for their studies.

Combining both biophysical and biochemical cues was hypothesized to better mimic the extracellular milieu of vascular endothelial cells. In fact, we observed a significant improvement in HUVEC adhesion and morphology on $2\mu\text{m}$ gratings+cRGD films from Unpatterned films. The gratings structures resemble the inherent fibrillary structure of the vascular endothelial basement membrane [37], suggesting activation of a cellular signaling cascade that stimulates physiological behavior. Another possible effect of topography is on surface wettability and concomitant protein adsorption [38]. For instance, we observed similar trends between water contact angle and cell adhesion on various modified surfaces. This is exemplified in $2\mu\text{m}$ gratings+cRGD films, which showed a significant decrease in wettability when compared with Unpatterned films. In contrast, $10\mu\text{m}$ gratings or $10\mu\text{m}$ gratings+cRGD films further increased wettability and lacked improvement in HUVEC adhesion. Our results match a previous study where increasing vertical feature size associated with reduced endothelial cell adhesion [38].

While we observed significant changes in HUVEC viability and adhesion on modified and unmodified substrates with $2\mu\text{m}$ gratings, further characterization of other EC behaviour relevant to endothelialization should be performed to verify the advantage of these modifications on PVA. Indeed, HUVEC viability on modified PVA may be better measured by using the more reproducible and sensitive luciferase-based assay that measures adenosine triphosphate levels [39]. Cell adhesion may also be further examined by measuring cell area and changes in focal adhesion size [40]. EC migration, an important contributor in trans-anastomotic migration, may also be studied. Long-term culture of ECs on PVA substrates should be done to give insight into EC phenotype or function [41]. These additional assays can be used to further verify the advantage of topographical modification on EC behaviour relevant to *in situ* graft endothelialization.

In addition to the known effects on endothelial cell behavior, topography has also been demonstrated to affect platelet adhesion in *in vitro* assays. Milleret *et al.* [42] have shown that increasing electrospun fiber diameter to 2–3 μm significantly enhanced thrombin generation and platelet adhesion. Interestingly, in this study the PVA films with 2 μm and 10 μm gratings topography had no statistical difference in platelet adhesion compared to Unpatterned films. The *in vitro* hemocompatibility assay performed on Unmodified and cRGD films with convex and concave structures showed a clear trend of increased absorbance and microparticle formation. This may be attributed to the retention of the protein conformation and activity on surfaces with high curvatures [43]. Nonetheless, SEM analysis of attached platelets showed a marked improvement in platelet morphology when films were modified with topography or both topography and cRGD, suggesting decreased platelet activation, reiterating the utility of combined biophysical and biochemical modification on PVA for vascular applications.

Although we observed a general trend of increasing platelet adhesion and activation using *in vitro* assays on patterned films (with or without cRGD) compared with Unpatterned films, the topographical cues on PVA are necessary to stimulate *in situ* endothelialization. Thus, we proceeded to study platelet adhesion using the *ex vivo* hemocompatibility assay on selected modifications that yielded relatively lower platelet adhesion and better HUVEC adhesion. While statistical differences were seen in the *in vitro* hemocompatibility studies between the various surface treatments, PVA grafts tested in the baboon shunt were equivalent. Overall, the minimal platelet accumulation and fibrin incorporation seen on the PVA grafts in the *ex vivo* shunt studies is very promising. As expected for arterial flow conditions, fibrin incorporation was low on all of the tested grafts. Platelet accumulation for all the PVA groups was equivalent to the clinical control, bare ePTFE, and the average accumulation seen at 60 mins on PVA was in the range of $0.07\text{--}0.36 \times 10^9$ platelets cm^{-1} . The maximum of this range of platelet adhesion on the PVA grafts, is lower than previous studies in our lab that examined endothelialized ePTFE grafts [27,44]. These grafts, seeded with baboon endothelial outgrowth cells, accumulated approximately 1×10^9 platelets cm^{-1} over 60 mins. While we and others feel that an endothelial layer is important for *in vivo* performance of any vascular device [45–47], preseeding of vascular devices is costly and time-consuming. Hemocompatibility and adhesive capacity of 2 μm gratings+cRGD PVA grafts may eliminate the need for preseeding and permit spontaneous endothelialization *in situ*.

While some of the trends remained between *in vitro* and *ex vivo* assays, the *ex vivo* shunt studies are likely more representative of clinical performance. It is a powerful model that uses whole blood without anticoagulant or anti-platelet therapies and examines thrombosis under hemodynamically relevant conditions. However, unlike the ePTFE controls from this study and others [27,44,48], which showed a plateau of platelet accumulation starting from 45 mins, the 2 μm gratings+cRGD and Unpatterned+cRGD PVA grafts started to increase their platelet accumulation rate around 45 mins. Future work with these grafts should examine longer blood exposure time to determine whether platelet accumulation reaches a plateau after 60 mins. Previous use of this model has tested grafts up to 4 hrs, showing a steady increase in platelet accumulation on bare ePTFE between 1 and 4 hrs [49].

Additionally, future work will also include *in vivo* studies to assess graft patency and endothelialization rates of the various PVA implants. The initial *ex vivo* shunt study supported the hypothesis that the PVA film and graft surfaces could be modified both biochemically and with physical patterning without deteriorating the hemocompatibility of the PVA grafts.

Endothelialization of modified PVA surfaces is an important contribution to the long-term patency of the vascular grafts. This study used HUVECs, which are a standard model for studying endothelial behaviour. In future studies, autologous endothelial cells, such as endothelial outgrowth cells [44], could be used to study the endothelial response on modified PVA surfaces in parallel with baboon *in vivo* implantation studies. The responses of venous and arterial endothelial cells to 2 μ m or larger gratings have been shown to be comparable [16]. Therefore, no differences are expected in the response of venous- and arterial-derived endothelial cells to topographically modified PVA vascular grafts. Additional graft implantation studies in an established animal model such as the baboon aorto-iliac model would give valuable insight in its patency, intimal hyperplasia and thrombosis, and extend its application to long-term blood contacting devices like hemodialysis grafts. Graft implantation studies will also be important for determining the beneficial effect of the chosen topographical and biochemical modification on endothelialization and thrombogenesis *in vivo*.

We have shown that PVA grafts have improved hemocompatibility and compliance from currently used clinical graft materials. These improvements may enhance long-term graft patency and clinical success for small-diameter vascular grafts. Additionally, the data presented here suggest that further improvements to the surface properties of the PVA have the potential to increase endothelial cell adhesion and proliferation. Providing these necessary stimuli to form an endothelium *in vivo* will be important to long-term biologic success.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Endothelial cell viability, adhesion and proliferation on poly(vinyl alcohol) (PVA) films modified with biochemical factors

(A) Endothelial cell viability is given as ratio of fluorescent signal from live (calcein-AM positive cells) over dead (Ethidium homodimer-1 positive cells) cells. No statistical differences were found between all groups. (B) Endothelial cell adhesion is changed on PVA films with biochemical modifications. Heparin films showed significant decrease in cell adhesion compared with Fibronectin, RGDS and cRGD films. (C) Endothelial cell proliferation is altered on PVA with biochemical modification. The proliferation rate is

given as the percentage of cells with EdU uptake over the total cell count. Groups separated by different letters are considered statistically different from 1-way ANOVA analysis.

Figure 2. *In vitro* blood compatibility of PVA films modified with biochemical cues

(A) In the LDH assay, absorbance correlates to the number of platelets attached to modified PVA films. Groups separated by different letters are considered statistically different from 1-way ANOVA analysis. (B) Platelet activation after contacting modified PVA films was measured by microparticle release. No statistical differences were found between all groups. (C) SEM images of the platelets adhered to the surface of the PVA films. Platelets attached on cRGD films had the least activated morphology.

Figure 3. The combination of topography and biochemical cues improves endothelial cell viability and adhesion

(A) Endothelial cell viability is given as a ratio of the number of live over dead cells. Cell viability significantly increased on 2µm gratings+cRGD, 10µm gratings+cRGD, Convex, Convex+cRGD, Concave and Concave+cRGD films compared to Unpatterned (without cRGD) films, as detected by 2-way ANOVA analysis. (B) Endothelial cell adhesion increased on 2µm gratings+cRGD films compared to Unpatterned (without cRGD) films. Groups separated by different letters are considered statistically different from a 2-way ANOVA analysis. (C) Endothelial cell morphology on modified PVA films. Red denotes actin fibers, blue denotes nucleus and white arrows denote grating axis. Scale bar = 20 µm.

Figure 4. Topographical cues and biochemical cues improve blood compatibility by decreasing platelet activation and adhesion

(A) In the LDH assay, absorbance correlates to the number of platelets attached to modified PVA films. No statistical differences were found between all groups. Groups separated by different letters are considered statistically significant using Kruskal-Wallis test. (B) Platelet activation was measured using microparticle release. Films of 2 μ m gratings+cRGD showed the largest decrease while Convex+cRGD showed the largest increase in number of microparticle formation compared with their respective patterned PVA controls. Groups separated by different letters are considered statistically different with a 2-way ANOVA. (C) SEM showed platelets had less activated morphology on PVA with topography compared to platelets on Unpatterned PVA or glass. The incorporation of cRGD into patterned films improved platelet morphology by indicating a less activated morphology.

Figure 5. *Ex vivo* platelet and fibrin accumulation over 60 min exposure to whole blood
 (A) Total platelets are shown per cm length of graft at 5 min intervals over 60 min. Statistical differences are shown for the repeated measures 1-way ANOVA. Groups separated by different letters are considered statistically different. (B) Total fibrin accumulation is shown per cm length. Statistical differences are indicated by different letters above the groups from a 1-way ANOVA.

Table 1
Summary of PVA modification with biomolecules

Amount of immobilized biomolecule is given in weight per gram of PVA and as a molar ratio of hydroxyl groups with respect to PVA.

Biomolecule	Molecular weight	Amount added per gram PVA	Ratio of hydroxyl groups per molecule PVA:biomolecule
Heparin	12–15 kDa	0.286 mg/g PVA	4.28:1
Fibronectin	26.24 kDa	28.57 µg/g PVA	1.51:1
RGDS	433.43 Da	28.57 µg/g PVA	600:1
cRGD	1008.18 Da	28.57 µg/g PVA	1200:1

Terminology for PVA scaffolds with the different combinations of biochemical and topographical modifications

PVA films modified with topography are referred to by the topography name and dimension (when necessary). PVA films modified with both topography and cRGD peptide are referred to as their topography name appended with “+cRGD”. PVA films without any biochemical modification or patterning are referred to as “Unmodified” or “Unpatterned,” respectively. PVA films with cRGD but without topography are referred to as “Unpatterned+cRGD”.

Table 2

Unmodified	Unpatterned	Gratings with 2 μm ridge $\times$ 2 μm width $\times$ 2 μm depth	Gratings with 10 μm ridge $\times$ 10 μm width $\times$ 10 μm depth	Convex microlens with 1.8 μm diameter, 2 μm pitch, 0.7 nm sag	Concave microlens with 1.8 μm diameter, 2 μm pitch, 0.7 nm sag
cRGD modification	Unmodified and Unpatterned control	2 μm gratings	10 μm gratings	Convex	Concave
	Unpatterned +cRGD control	2 μm gratings+cRGD	10 μm gratings+cRGD	Convex+cRGD	Concave+cRGD

Table 3**Surface analysis of biochemically modified PVA**

Water contact angle data that are statistically different are separated by different letters.

PVA modification	Elemental analysis [‡]				Water contact angle (°)
	N (%)	O (%)	C (%)	O/C ratio	
Unmodified	ND	30.4	69.6	0.44	ND
cRGD	0.76	29.3	68.8	0.43	0.01
Fibronectin	0.70	30.3	69.0	0.44	0.01
Heparin	0.78	32.3	66.9	0.48	0.01

ND = non detectable.

[‡]The balance in XPS for untreated and plasma-treated PVA films were Si, P. Statistically different data are separated by different letters.

Table 4
Water contact angle of patterned PVA films with and without cRGD modification

Data that are statistically different are separated by different letters.

	2µm gratings	10µm gratings	Convex	Concave
Unmodified	34.1 ± 2.29 ^{*†B}	13.4 ± 2.29 ^{*†F}	19.6 ± 3.16 ^{†DEF}	26.9 ± 1.75 ^{†BCD}
+cRGD	49.0 ± 3.24 ^{*A}	16.4 ± 1.11 ^{*†EF}	30.8 ± 4.66 ^{†BC}	25.2 ± 2.00 ^{†CDE}

* denotes statistical significance against Unpatterned, which is found in Table 3.

† denotes statistical significance against Unpatterned+cRGD, which is found in Table 3.