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Platelet and endothelial adhesion on fluorosurfactant polymers designed for vascular graft modification

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Abstract

A prominent failure mechanism of small-diameter expanded polytetrafluoroethylene (ePTFE) vascular grafts is platelet-mediated thrombosis. We have designed surface modification for ePTFE consisting of a self-assembling fluorosurfactant polymer (FSP) bearing biologically active ligands, including adhesive peptides and polysaccharide moieties. The goal of this biomimetic construct is to improve graft hemocompatibility by promoting rapid surface endothelialization, while minimizing platelet adhesion. Here, we present a direct comparison of platelet and endothelial cell (EC) adhesion to FSPs presenting one of three cell adhesion peptides: cyclic **Arg-Gly-Asp**-D-Phe-Glu (cRGD), cyclic *Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys* (cRRE, *disulfide bond cyclization), linear Gly-**Arg-Gly-Asp**-Ser-Pro-Ala (RGD) or a polysaccharide moiety: oligomaltose (M-7), the later designed to prevent plasma protein adhesion. Measurements of soluble peptide-integrin binding indicated that cRRE exhibits the least affinity for the $\alpha_{IIb}\beta_3$ platelet fibrinogen receptor. Analysis of static and dynamic platelet adhesion on FSP modified surfaces demonstrated that both M-7 and cRRE promote significantly less platelet adhesion compared with RGD and cRGD FSPs, while EC adhesion was similar on all peptide FSPs and minimal on M-7 FSP. These results illustrate the potential for ligands presented in a FSP surface modification to selectively adhere ECs with limited platelet attachment.

Introduction

More than half a million coronary and peripheral vascular bypass procedures are performed in developed nations every year [1]. For these procedures, the replacement conduit type of choice is an autologous vessel, which is often limited by vessel availability [2]. Alternative polymer conduits, including expanded polytetrafluoroethylene (ePTFE), exhibit high failure rates due to thrombosis and intimal hyperplasia, especially in small-diameter (<5 mm) applications [2]. As a result, there is a need for a functional and readily available small-diameter synthetic graft with long-term patency.

A critical early step in synthetic graft failure is platelet adhesion and thrombosis. Platelets play an important role in hemostasis, inflammation, and wound healing [3–5]. To reduce platelet interactions, reported surface modifications have included grafting polysaccharides or hydrophilic polymers to suppress platelet and protein adhesion [6, 7]. Another approach is the luminal attachment of endothelial cells (ECs) to enhance graft hemocompatibility through their native anticoagulative, antiplatelet, and fibrinolytic functions[2, 8, 9].

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To address the failure of synthetic grafts to endothelialize in humans, a variety of seeding methods have been investigated [10]. However, most seeding methods are impractical in emergency and smaller hospital settings as they require prolonged cell culture within sterile facilities [1, 11]. As such, the development of substrates bearing cell-binding peptides that promote *in vivo* endothelialization are being pursued [1, 12]. However, following implantation, exposed surface ligands intended to promote EC adhesion often bind platelets, thereby exacerbating surface thrombogenicity [13, 14].

To promote EC with limited platelet adhesion, we have developed fluorosurfactant polymer (FSP) surface modifications, which consist of three components: a poly(vinyl amine) backbone, fluorocarbon branches for stable adsorption, and a functional ligand such as a cell-binding peptide or polysaccharide to prevent non-specific adhesion (Fig. 1) [15, 16]. Utilization of FSPs for surface modification of ePTFE allows for enhanced control over ligand orientation and spacing unavailable in plasma or photochemical treatment, avoidance of potentially pathogenic or immunoactive biological materials inherent in the incorporation of ECM proteins, and stability under shear stress and sterilization [15, 17]. In this study, we investigated the initial platelet and EC attachment to three peptide FSPs that present different integrin-binding peptides: linear Gly-**Arg-Gly-Asp**-Ser-Pro-Ala (RGD), cyclized **Arg-Gly-Asp**-D-Phe-Glu (cRGD), and cyclized *Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys* (cRRE, * denotes disulfide bond cyclization) (Fig. 1c) or a polysaccharide moiety: oligomaltose (7-mer, M-7). Past research has demonstrated that surfaces presenting RGD [15], cRGD [18], or cRRE [19] promote EC adhesion. In this paper, we aim to build upon current knowledge in peptide and sugar presenting graft modifications through the direct comparison of platelet and EC interactions on the modified surfaces.

Materials and Methods

Cyclic RGD Peptide Synthesis

The cell adhesive peptide cyclic(RGDfE)-SSSK (cRGD) was synthesized using a PerSeptive Biosystems (model 9050) solid-phase peptide synthesizer (Applied Biosystems) via 9-fluorenylmethoxycarbonyl (Fmoc) methodology. Fmoc-protected amino acids (AnaSpec), including a glutamic acid residue modified by an α -allyl protected carboxy terminus, were incorporated onto a Fmoc-PAL-PEG-PS resin (0.36 mmol/g, PerSeptive Biosystems) beginning with lysine. Following synthesis of the linear peptide, the α -allyl group was removed as described by Delforge et al. [20]. The N-terminal Fmoc protecting group was then cleaved and peptide cyclization was performed on the column through the addition of 2 equivalents of O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and diisopropylethanolamine. The cyclic peptide was then deprotected and cleaved from the resin followed by preparatory scale high performance liquid chromatography purification. The final peptide product was characterized by mass-spectroscopy: single major peak at $m/z=994.6$ and $^1\text{H-NMR}$ in D_2O : 7–7.3 ppm (benzyl protons) and 1.2–5.2 ppm (CH_2 and peptide side group protons).

To verify cRGD peptide structure, mass spectroscopy confirmed accurate molecular weights for the α -allyl, Fmoc-protected peptide (1,274.4), peptide following α -allyl deprotection (1,234.3), peptide following N-terminal Fmoc removal (1,011), and cRGD peptide following cyclization (994).

Cyclic RGD FSP Synthesis

The synthesis of cRGD FSP was performed as previously described [15, 19, 21]. Briefly, cleaved and purified cRGD (50 mg, 0.051 mmol) was functionalized at the lysine ϵ -amino group to glutaraldehyde (25 mg, 0.25 mmol) with a NaCNBH_3 (3.24 mg, 0.051 mmol)

catalyst. $^1\text{H-NMR}$ in dimethyl sulfoxide (DMSO-d_6) confirmed successful peptide-aldehyde conjugation: 9.7 ppm (CHO), 7–8.5 ppm (amide backbone and benzyls protons), and peaks in 1.2–5.2 ppm (CH_2 and peptide side group protons).

To conjugate peptide to PVAm, glutaraldehyde-modified peptide (31 mg, 0.029 mmol) was reacted with PVAm (2.08 mg, 0.048 mmol) via a Schiff base reaction. Successful attachment was confirmed by IR (KBr , cm^{-1}): 3305 (ν (OH)), 2926–2968 (ν (CH) of CH_2 and CH), 1650 (amide-I) and 1547 (amide-II) and $^1\text{H-NMR}$ in DMSO-d_6 : no residual aldehyde peak (9.7 ppm).

To attach fluorocarbon branches, N-perfluoroundecanoyloxy succinimide (3.48 mg, 0.0054 mmol) was reacted with PVAm-cRGD (18.4 mg). Successful attachment was confirmed by IR: 3305 (ν (OH)), 2926–2968 (ν (CH) of CH_2 and CH), 1650 (amide-I), 1547 (amide-II), 1224 and 1154 (CF_2).

RGD and cRRE FSP Synthesis

Synthesis of RGD [15] and cRRE FSPs [19] were conducted as previously described. Briefly, the peptides Gly-Ser-Ser-Ser-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys and Gly-Ser-Ser-Ser-Arg-Gly-Asp were synthesized via solid phase peptide synthesis. Cysteins on linear Gly-Ser-Ser-Ser-Cys-Arg-Arg-Glu-Thr-Ala-Trp-Ala-Cys were cyclized to form GSSS-*CRRETAWAC* (*denotes disulfide bond) via solution oxidation. Peptides, and subsequently, N-perfluoroundecanoyloxy succinimide were conjugate to PVAm through Schiff base reactions. Final and intermediate products were characterized by mass spectrometry, IR, and $^1\text{H-NMR}$.

M-7 FSP Synthesis

The reducing end of oligomaltose (7 glucose residues, M-7, Sigma) was converted to a lactone as previously described [22]. Malto lactone (67 mg, 0.052 mmol) in 3 ml DMSO was reacted with PVAm (5 mg in 1 ml) methanol and stirred for 48 h at 75°C. The product was purified by dialysis against water using Spectra/Por 3 regenerated cellulose membranes and lyophilized (yield ~70%). To confirm attachment of PVAm to maltose, the product was characterized by IR: 3305 cm^{-1} (ν (OH)), 2926–2868 cm^{-1} (ν (CH) of CH_2 and CH), 1650 cm^{-1} (amide-I), 1547 cm^{-1} (amide-II), and 1149–1032 cm^{-1} (ν (C-O)) and $^1\text{H-NMR}$ in DMSO-d_6 : 1.0–1.6 ppm (CH_2 from PVAm backbone), 3.0–4.1 ppm (CH of PVAm backbone and CH and CH_2 of oligomaltose excluding glycosidic linkages), 4.1–5.3 ppm (oligosaccharide OH's and CH of glycosidic linkages), 7.2–8 ppm (NH of amide linkage).

To attach fluorocarbons to the PVAm backbone, N-perfluoroundecanoic acid (16.36 mg, 0.029 mmol) was added to PVAm-(M-7) (40 mg) in 3 ml DMSO with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (55.4 mg, 0.29 mmol) and N-hydroxysuccinimide (33.3 mg, 0.29 mmol) with agitation at room temperature for 24 h. M-7 FSP was acetone precipitated then purified by extensive dialysis against water and lyophilized (yield ~68%). The product was characterized by IR: 3305 (ν (OH)), 2926–2968 (ν (CH) of CH_2 and CH), 1650 (amide-I) and 1547 (amide-II), 1224 and 1154 (CF_2), 1149–1032 (ν (C-O)).

Soluble Peptide Synthesis

Peptides used for soluble peptide studies were synthesized as described above without the GSSS- or KSSS- spacers. As inclusion of either lysine or glutamic acid as the fifth residue in cRGD results in the same integrin binding behavior [23, 24], two cRGD sequences: cyclic(RGDfK) (Peptide's International) and cyclic(RGDfE) were utilized in soluble peptide studies.

Endothelial Cell Culture

Human pulmonary ECs (Cambrex) were cultured on fibronectin (FN, sigma, 1 $\mu\text{g}/\text{cm}^2$) coated tissue culture polystyrene flasks (Costar) in complete growth media as previously described [15]. Passage 7 ECs were used in all experiments.

Blood Collection and Cell Suspension Preparation

Venous blood was drawn from healthy, aspirin-refraining adult donors in compliance with protocols approved by the Institutional Review Board for Human Investigations at Case Western Reserve University. Blood was drawn into 3.2% sodium citrate anticoagulant (Sigma) in a ratio of 9:1.

To prepare washed platelet suspension (WPS), blood was centrifuged at 150g for 15 min. The platelet rich plasma (PRP) layer was removed and centrifuged at 1800g for 10 min to pellet platelets. The supernatant platelet poor plasma (PPP) was removed and the platelet pellet was resuspended in phosphate-buffered saline (PBS) with 1.8 mM sodium EDTA (Sigma). The solution was then centrifuged at 1800g for 10 min followed by suspension in 1% bovine serum albumin (BSA, Sigma) at a final concentration of $\sim 50,000$ platelets/ μl as measured by an AcTDiff Coulter Counter (Beckman Coulter).

To prepare washed platelet-endothelial cell suspensions (WPES), confluent ECs were detached via 30 min incubation in Cellstripper (Mediatech) and suspended in 1% BSA. ECs were quantified via hemocytometer and mixed with platelets in 1% BSA to a final concentration of $\sim 50,000$ ECs/ cm^2 (190 ECs/ μl) and $\sim 50,000$ platelets/ μl .

Flow Cytometry

Flow cytometry was used to assess p-selectin (CD41a) expression, a marker of platelet activation, was performed by Cleveland Clinic Flow Cytometry labs following standard protocols [25] using fluorescein isothiocyanate (FITC) anti-CD41a and phycoerythrin anti-CD62p.

Solid-phase Integrin Binding Assay

A solid-phase integrin binding assay was performed as previously described [19]. Briefly, 5 $\mu\text{g}/\text{ml}$ purified $\alpha_{\text{IIb}}\beta_3$ integrin (Enzyme Research Laboratories) was adsorbed overnight onto 96 well plates (Fisher). Wells were then blocked with BSA and incubated with 10 $\mu\text{g}/\text{ml}$ fibrinogen (FG, Enzyme Research Laboratories) alone and with varying concentrations of soluble peptide. Bound FG was detected by incubation with a primary peroxidase conjugated FG antibody (Enzyme Research Laboratories) followed by development with 3,3',5,5'-Tetramethylbenzidine (Calbiochem). Normalized FG binding was determined by dividing absorbance at 650 nm from peptide-FG competition to absorbance from control wells containing FG only. IC_{50} values for inhibition of FG binding were determined by a nonlinear regression curve fit of 60 data points/peptide (≥ 11 concentrations/peptide with ≥ 4 replicates/concentration) using Origin 7.5 data analysis software (OriginLab Corporation). The coefficients of determination (R^2) for cRRE, cRGD, and RGD regression fits were 0.92, 0.97, and 0.83, respectively.

Peptide Inhibition of Platelet Aggregation

Peptide inhibition of platelet aggregation was performed as previously described [19]. Platelet aggregation in a stirred suspension consisting of PRP, 10 μM adenosine-5'-diphosphate (ADP, Bio/Data Corporation), and various concentrations of soluble cRRE, RGD, or cRGD (both cRGD sequences Arg-Gly-Asp-D-Phe-**Glu** and Arg-Gly-Asp-D-Phe-**Lys**) was measured for 5 min via a PAP-4 aggregometer (Bio/Data Corporation). To

calculate percent inhibition of platelet aggregation, maximum aggregation observed for each sample was normalized to a control peptide-free PRP suspension. IC_{50} values for platelet aggregation inhibition were determined by nonlinear regression curve fit of ≥ 15 data points/peptide (≥ 5 concentrations/peptide with ≥ 3 replicates/concentration) using Origin 7.5 data analysis software. R^2 values for cRRE, cRGD, and RGD regression fits were 0.84, 0.88, and 0.83, respectively.

Surface Preparation

FSPs were dissolved in water (2 mg/ml) and allowed to adsorb on fluorosilane-self assembled monolayers (FSAM, prepared as described [15]) for 24 h. Surfaces were then removed from solution, rinsed with water, and allowed to dry for 24 h. Surface modification was confirmed with advancing and receding water contact angles measured by a Rame-Hart contact angle goniometer ($n \geq 6$) via the sessile drop method [21].

FN coated glass surfaces were prepared immediately before use by incubating human FN (1 $\mu\text{g}/\text{cm}^2$) on glass coverslips for ~ 1 h.

Static Adhesion Assay

Before use, all test surfaces were incubated with 2% BSA for 30 min, and all cell suspensions were supplemented with CaCl_2 and MgCl_2 to a final concentration of 2 mM and 1 mM, respectively. Surfaces were statically incubated in WPS ($n=4$) or WPES ($n=3$) for 30 min at 37°C .

Rotating Disk Assay

A rotating disk system (RDS) was employed to expose surfaces ($n=3$) to WPS in a reproducible shear stress environment [6]. Using this apparatus, shear stress (τ_s) is a function of radial distance: $\tau_s = 0.8 * \eta * r * \omega^{1.5} * \nu^{-0.5}$. Where ν =kinematic viscosity, ω =angular velocity, and η =absolute viscosity.

For WPS, ν was measured to be 0.00749 Stokes using an Ostwald viscometer (Barnstead International). Density (ρ) was measured to be 1.002 g/ml via a XS 105 analytical balance (Mettler Toledo) and used to calculate ω ($\omega = \nu * \rho = 0.00750$). All surfaces were rotated in WPS for 1 h at 37°C at a shear stress range of 0–40 dynes/ cm^2 .

Adhesion Quantification

Following exposure to cells, all surfaces were washed with PBS and fixed with 4% paraformaldehyde (PFA, Sigma) for 20 min. Surfaces exposed to platelets were stained in the dark with a solution of FITC anti-CD41a (anti-GP IIIa, 1:100 dilution, BD Biosciences) in 1% FBS with addition of Alexa Fluor 568 phalloidin (actin cytoskeleton, 1:40 dilution, Molecular Probes) and 1 μM 4',6-diamidino-2-phenylindol, dihydrochloride (nuclear stain, Molecular Probes) for surfaces exposed to both platelets and ECs.

Fluorescently labeled cells were visualized by epifluorescent illumination on a Nikon Diaphot 200 with 10x and 40x objectives. For static adhesion, 14–33 fields/surface were imaged, while for dynamic adhesion, 8 fields/surface were imaged at each shear stress value (10 radial distances on 4 axes). EC and platelet adhesion were quantified using ImageJ (NIH) image analysis software. Statistical analysis was performed in Microsoft Excel 2003 using a two sample *t*-test with significant differences taken to be at *p*-values < 0.05 .

Results

Platelet Activation

Flow cytometry of platelets in PRP and WPS using antibodies for p-selectin (CD62p) and the constitutively expressed integrin subunit α_{IIb} (CD41a) receptors indicated that PRP preparations exhibited 32.1% p-selectin expression compared to 73.9% found in WPS. Platelet activation in PRP and WPS was therefore taken to be 32.1% and 73.9%, respectively.

Soluble Peptide-platelet Interaction

The relative affinity of free peptide for purified platelet $\alpha_{IIb}\beta_3$ integrin receptor was tested through a solid-phase integrin binding assay. As both FG and soluble peptides compete for a constant number of binding sites, peptides that possess higher affinities for $\alpha_{IIb}\beta_3$ will cause a greater decrease in FG binding and exhibit correspondingly lower IC_{50} values. Results from this assay (Table 1) indicate that cRRE affinity for purified $\alpha_{IIb}\beta_3$ ($IC_{50}=3,054\pm90$ μ M) [19] is two orders of magnitude lower than either RGD ($IC_{50}=52\pm6$ μ M) [19] or cRGD ($IC_{50}=25\pm2$ μ M).

Soluble peptide affinity for platelet membrane presented $\alpha_{IIb}\beta_3$ was measured via a platelet aggregation inhibition assay. Peptide inhibition of platelet aggregation is positively related to affinity for receptors involved in aggregation (most prominent of which is $\alpha_{IIb}\beta_3$ [26]) resulting in a relation between a lower IC_{50} value and higher $\alpha_{IIb}\beta_3$ binding affinity. Results from this assay (Table 1) followed a similar trend to the solid-phase integrin-binding assay, where cRRE affinity for platelet receptors ($IC_{50}=2,700\pm390$ μ M) [19] was an order of magnitude lower than RGD ($IC_{50}=280\pm40$ μ M) [19] or cRGD ($IC_{50}=173\pm18$ μ M).

Surface Characterization

To confirm successful surface modification, significant reductions in water contact angle were observed following modification on cRGD, M-7 (Table 2), RGD [15], and cRRE [19] FSPs compared to FSAM.

Static Platelet Adhesion and Morphology on Fluorosurfactant Polymers

Platelet adhesion on M-7 FSP (0.20 normalized to FN) and cRRE (0.35) was found to be significantly lower ($p<0.01$) compared with FN while that on RGD (1.40) and cRGD (1.50) was found to be significantly greater ($p<0.01$) (Fig. 2a).

A relative measure of platelet vs. EC adhesion was obtained via a competitive platelet-EC static adhesion assay. EC adhesion on cRRE (0.93), RGD (0.99), and cRGD (1.05) FSPs was comparable while that on M-7 FSP (0.38) was significantly lower ($p<0.01$) than FN (Fig. 3a). Platelet adhesion following static exposure to platelet-EC suspensions revealed that significantly less ($p<0.01$) platelets adhered to M-7 FSP (0.51) compared with FN while significantly more ($p<0.01$) platelets were adherent to RGD (1.99) and cRGD (2.66) FSPs (Fig. 3a). Fluorescent images indicated that platelets adhered primarily to exposed surfaces between attached ECs with little adhesion on EC membranes (Fig. 3b).

A high proportion of platelets exhibited an activated, spread morphology on RGD and cRGD FSPs (Fig. 2b, 3b) with an intermediate proportion on cRRE FSP and FN and a low proportion on M-7 FSP. The majority of adherent platelets on M-7 FSP were rounded with minimal cytoplasmic extensions. Adherent ECs on FN and peptide FSPs were similarly spread while those on M-7 FSP were less spread with an indistinct cytoskeleton (Fig. 3b).

Dynamic Platelet Interaction with Fluorosurfactant Polymers

A dynamic adhesion assay was employed to assess platelet adhesion in a shear stress environment. On all surfaces, as shear stress increased up to 40 dynes/cm², percent platelet surface coverage decreased (Fig. 4a). RGD and cRGD FSPs exhibited the highest degree of platelet surface coverage (max coverage=50–80%), followed by FN (max coverage=30%), and the lowest degrees of platelet coverage were observed on cRRE and M-7 FSPs (max coverage=5–15%).

Discussion

A major regulator of platelet and EC adhesion is the integrin class of transmembrane receptors [27, 28]. On platelets, the $\alpha_{IIb}\beta_3$ integrin is the most numerous receptor and plays a critical role in mediating platelet adhesion, activation, aggregation and thrombosis through interactions with ligands including collagen, FG, and FN [27]. On the other hand, in ECs, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ are the predominant integrin receptors important in mediating cell survival and adhesion [29, 30]. A central goal for vascular graft surface modifications designed to facilitate *in vivo* endothelialization is the promotion of EC attachment while minimizing platelet adhesion. To accomplish this, surface ligands should predominately interact with EC adhesion receptors such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$ and not platelet adhesion receptors such as $\alpha_{IIb}\beta_3$.

A number of peptide sequences have been investigated to promote binding to integrin receptors including Arg-Gly-Asp (RGD). RGD is a critical peptide sequence found in proteins such as FG, laminin, and FN and mediates binding of these ligands with integrin receptors including $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_{IIb}\beta_3$ [13]. However, cell types ranging from osteoblasts to platelets have been shown to adhere on RGD presenting surfaces [13]. Through cyclization and induction of angular strain, derivatives of RGD have been synthesized with differing affinities for integrin receptors [31]. One such peptide is cRGD which exhibits enhanced affinity for the EC $\alpha_v\beta_3$ [32]. Finally, the peptide cRRE [33], initially identified via phage display library, exhibits greater affinity for the EC $\alpha_5\beta_1$ [33].

Comparison of soluble RGD, cRGD, and cRRE affinity for platelet ($\alpha_{IIb}\beta_3$) and EC ($\alpha_v\beta_3$ and $\alpha_5\beta_1$) integrins reveal that all three peptides possess similar affinity for $\alpha_5\beta_1$, but cRRE exhibits at least two-fold lower affinity for $\alpha_{IIb}\beta_3$ than either RGD peptide (Table 3). Compared to linear RGD, which has a similar affinity for all three integrins, cRGD has improved affinity for $\alpha_v\beta_3$. A possible explanation for differences in relative peptide affinities for integrin binding is the interaction with specific α and β integrin subunits. Studies into the mechanism of RGD and cRGD interaction with integrin receptors have revealed that both peptides predominantly interact with the integrin β -subunit [31, 34]. Since a variety of different integrins possess the same β -subunit, (e.g. $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$), presenting RGD-derived peptides may be insufficient to promote EC over platelet adhesion. In nature, integrin-binding specificity in RGD-containing proteins is mediated by the complementary interactions of synergy sequences such as FN's Pro-His-Ser-Arg-Asn (PHSRN) with the integrin α -subunit in conjugation with interactions between RGD and the β -subunit [35, 36]. Unlike both RGD and cRGD, cRRE's Trp residues interact with the Trp¹⁵⁷ residue on the α_5 -subunit [37] in addition to extensive interactions with the β_1 -subunit through the Arg-Arg-Glu sequence [31]. As a result, the mechanism of peptide-integrin binding mediated by cRRE may be more suited to promote selective integrin-binding compared to either RGD or cRGD.

Platelet surface adhesion involves the complex interplay of a variety of different adhesion receptors, such as the von Willebrand receptor (GPIb/V/IX), the Fc gamma receptor, and collagen receptor ($\alpha_2\beta_1$), in addition to $\alpha_{IIb}\beta_3$ [28]. It is therefore important to study platelet

adhesion onto FSP modified surfaces as this allows a more comprehensive evaluation of platelet interactions.

Platelet static adhesion can be considered a “worst case” scenario that allows for the prolonged, undisturbed formation of platelet-surface interactions [38] while dynamic adhesion allows for the observation of adhesion within a shear stress environment. In addition, washed platelets were employed in all assays as the use of such a highly activated platelet suspension (as indicated by flow cytometry, see materials and methods) provided a more stringent test of platelet-surface interactions. Under all conditions, platelet adhesion onto peptide FSPs exhibited a trend in platelet-surface reactivity which correlated with soluble peptide affinity data, with cRRE FSP promoting lower platelet adhesion than either RGD or cRGD FSPs (Fig. 2a, 3a, 4a).

In general, the degree of platelet spreading observed is an indication of the extent of adherent platelet interactions. A spread and flattened platelet morphology is indicative of extensive, stable interactions and platelet activation [39, 40]. Analysis of platelet morphology on surfaces following static exposure to platelets alone (Fig. 2a) or to both platelets and ECs (Fig. 3b) suggest a high degree of platelet interaction and activation on RGD and cRGD FSPs, intermediate levels on FN coated glass and cRRE FSP, and lowest levels on M-7 FSP. These morphological differences are indicative of a higher degree of platelet activation on RGD and cRGD FSPs and may translate into enhanced stability of spread platelets to shear stress. This offers a possible explanation of why RGD and cRGD surfaces, which exhibited the highest proportion of spread platelets following static adhesion, promoted the highest platelet adhesion under shear stress (Fig. 4a). In contrast, cRRE and M-7 FSPs, which exhibited proportionally less spread platelets also promoted less platelet adhesion under shear stress (Fig. 4a).

Differences in cell adhesion are heavily dependent on surface ligand density and affinity. Since FSPs possess the same polymer backbone and identical peptide attachment chemistry, it is likely that ligand density varied little between FSP surfaces. On the other hand, whole proteins, such as FN, are considerably larger than peptides. Therefore, surfaces modified by protein adsorption likely exhibit lower ligand densities [15, 19, 41–43]. As such, higher ligand densities on FSP modified surfaces may account for the greater platelet coverage observed on RGD and cRGD FSPs compared with FN. Since cRRE ligand density is approximately equivalent to RGD and cRGD FSPs and higher than FN, the lower affinity of cRRE peptide for platelet adhesion receptors, and not differences in ligand density, offers a plausible explanation for the lower platelet coverage on cRRE FSP compared with RGD and cRGD FSPs.

Unlike platelets, ECs exhibit a different complement of adhesion receptors, most notably higher expression of $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which mediate interactions onto RGD [13], cRGD [18], cRRE [19], and FN [44] presenting surfaces. It is possible that each of these surfaces possess a “critical” ligand density, above which additional ligands do not increase initial EC attachment above a “saturation” level. Such a “critical” density was recently observed by Tugulu et al. [45] and may account for the similar levels of EC adhesion observed on peptide FSPs and FN. Previous studies have also indicated an antithrombotic EC phenotype on RGD [15] and cRRE [19] FSP surfaces.

Finally, both platelet and EC attachment are significantly less on M-7 FSPs compared to other surfaces. Surfaces modified by M-7 FSP do not possess ligands that mediate specific interaction with EC or platelet adhesion receptors. On the contrary, surface affixed maltose moieties act to inhibit surface adhesion through steric inhibition and enhancement of surface hydration [6, 7, 46]. Morphology of adherent platelets and ECs on M-7 FSP were also

indicative of a low degree of surface interactions as the few adherent cells were rounded and exhibited poorly developed actin cytoskeletons (Fig. 2b, 3b).

Conclusions

Platelet and EC adhesion and morphology were evaluated on biofunctional FSP modifications that presented RGD, cRGD, or cRRE peptides to promote specific cell attachment or the polysaccharide M-7 to inhibit non-specific adhesion. Of these peptides, cRRE exhibited the lowest affinity for platelet $\alpha_{IIb}\beta_3$ integrin. This translated into lower platelet adhesion on cRRE FSP compared to either RGD or cRGD FSPs under all conditions tested while EC attachment was comparable. On the contrary, M-7 FSP reduced both platelet and EC interactions under all conditions tested. These results highlight the feasibility of cRRE FSP for the improvement of graft biocompatibility through *in vivo* endothelialization strategies and M-7 FSP to reduce platelet adhesion.

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Figure 1.

Peptide FSP model and chemical structures. **A)** Chemical structure of peptide FSP. PEPTIDE indicates position of cell adhesion peptide (PEPTIDE= cRRE, RGD, or cRGD). Identity of amino group on spacer sequence is either glycine (G, for PEPTIDE=cRRE or RGD) or lysine (K, for PEPTIDE=cRGD). **B)** Molecular model of RGD FSP. Chemical structures of **C)** cRRE, **D)** RGD, and **E)** cRGD peptides.

Figure 2. Platelet static adhesion on experimental surfaces. **A)** Platelet surface coverage on test surfaces following static exposure to a washed platelet suspension (normalized to FN, FN platelet coverage=1). Error bars indicate standard deviation. †Significantly lower or *significantly higher (p<0.01) platelet adhesion compared to compared to FN. **B)** Fluorescent microscopy images of adherent platelets on experimental surfaces. Scale bar indicates 30 μm.

Figure 3. Competitive platelet-EC static adhesion on experimental surfaces. **A)** EC population (■) and %platelet surface coverage (□) on test surfaces following static exposure to a washed suspension containing both ECs (190 EC/μl) and platelets (50,000 platelet/μl) (normalized to FN, FN EC and platelet coverage=1). Error bars indicate standard deviation. †Significantly lower ($p<0.01$) EC adhesion compared to FN, and *significantly lower or ‡significantly higher ($p<0.01$) platelet adhesion compared to FN. **B)** Fluorescent microscopy images of adherent ECs and platelets on experimental surfaces. Scale bar indicates 50 μm.

Figure 4. Platelet dynamic adhesion on experimental surfaces. **A)** Percent platelet surface coverage on test surfaces following dynamic exposure to platelets via a rotating disk apparatus. **B)** Fluorescent microscopy images of surface adherent platelets exposed to 0, 7.2, and 14.9 dynes/cm² (vertical axis) on experimental surfaces (horizontal axis). Scale bar indicates 30 μ m.

Table 1
IC₅₀ values for peptide interactions

Concentration of free peptide corresponding to 50% inhibition (IC₅₀) of fibrinogen binding to surface immobilized $\alpha_{\text{IIb}}\beta_3$ integrin (left column) and to 50% inhibition of platelet aggregation (right column).

Peptide	Fibrinogen- $\alpha_{\text{IIb}}\beta_3$ Binding IC ₅₀ (μM)	Platelet Aggregation IC ₅₀ (μM)
cRRE	3,050 $\pm$ 90	2,700 $\pm$ 390
RGD	52 $\pm$ 6	280 $\pm$ 40
cRGD	25 $\pm$ 2	173 $\pm$ 18

Table 2
receding and advancing water contact angles

Water contact angle measurements on unmodified FSAM glass, cRGD FSP, and M-7 FSP. θ_a indicates highest advancing contact angle $\pm$ standard deviation while θ_r indicates lowest receding water contact angle $\pm$ standard deviation both from ≥ 6 measurements.

Substrate	θ_a	θ_r
FSAM	105 ± 2	95 ± 2
cRGD FSP	15.2 ± 8.5	4.8 ± 2.0
M-7 FSP	63.3 ± 2.8	11.9 ± 2.8

Table 3
IC₅₀ values for inhibition of integrin binding

Concentration of free peptide corresponding to 50% inhibition of soluble ligand binding to surface immobilized integrin receptors (IC₅₀, vitronectin- $\alpha_V\beta_3$, fibronectin- $\alpha_5\beta_1$, fibrinogen- $\alpha_{IIb}\beta_3$).

Integrin	cRRE	RGD	cRGD	Ref.
$\alpha_{IIb}\beta_3$	3,054	52	25	
$\alpha_V\beta_3$	>1000	0.32	0.05	[45] [32]
$\alpha_5\beta_1$	~1	7.7	6.4	[45] [32]