EXTRACELLULAR MATRIX BIOMIMICRY FOR THE
ENDOTHELIALIZATION OF CARDIOVASCULAR MATERIALS

by

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Eric Hugo Anderson

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TO MY DAUGHTER
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Extracellular Matrix Biomimicry for the Endothelialization of Cardiovascular Materials

Abstract

by

ERIC HUGO ANDERSON

A common reason for cardiovascular device failure is biomaterial surface-induced thrombosis. The body's natural defense against this problem is the glycocalyx of endothelial cells (EC) that line the interior of blood vessels. This interface represents the ideal nonthrombogenic surface. The glycocalyx contains a dense array of hydrated polysaccharides and glycoproteins, which combats nonspecific protein adsorption and controls specific interactions. A layer of endothelium on a cardiovascular implant would be most beneficial; however, EC can't attach and proliferate on the hydrophobic surface. To address this problem, novel surfactant polymers were developed to mimic the extracellular matrix and function as an interface between the material surface and EC. These surfactant polymers consist of functional cell-adhesive hydrophilic peptides and alkyl chains for adsorption onto the hydrophobic material coupled to a poly(vinyl amine) (PVAm) backbone. Surfactant polymers are synthesized and characterized at each step by mass spectrometry, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and high-pressure liquid chromatography. Hydrophobic surfaces are prepared and coated with the surfactant polymers followed by surface characterization using contact angle goniometry and AFM. Finally, EC are seeded onto modified surfaces, and attachment and growth are analyzed.
A series of surfactant polymers were synthesized by incorporating varying amounts of arginine-glycine-aspartic acid (RGD) peptide on the PVAm backbone. Dextran oligosaccharides were added to keep the hydrophilic:hydrophobic balance constant. Attachment and growth of EC on surfaces with the most RGD peptides was comparable to the fibronectin positive control. Cell adhesion decreased dramatically with decreasing peptide density. Surfactant polymers containing heparin-binding peptides (HBP, interact with EC heparan sulfate proteoglycans) were also synthesized. Compared to fibronectin, EC on HBP surfaces showed more focal adhesions at 3 hours, but significantly less growth over 48 hours. Surfaces with both HBP and RGD showed better cell growth compared to surfaces with RGD alone. Other peptide ligands were synthesized for increased affinity: a cyclic RGD peptide (mimics native loop conformation in fibronectin) and an RGD peptide tethered to the synergy PHSRN peptide using a PEO chain. In conclusion, cell adhesive surfactant polymers can promote EC attachment and growth and would serve as viable coatings for vascular materials.
Chapter 1. GENERAL INTRODUCTION

1.1. Current Status of Cardiovascular Biomaterials – Brief Overview

Atherosclerosis is a common disease, affecting many individuals and marked by gradual stenosis of the blood vessels. These stenotic vessels manifest smooth muscle cell proliferation, thrombus formation, and deposits of calcium, lipoproteins, and cholesterol. Ultimately, the vessel can become completely occluded, or a fragment of the occlusion may dislodge, possibly causing a blockage somewhere else in the body. Ultimately, the vessel must be replaced. These widespread procedures consume over 1 million vascular grafts each year [1.1]. Synthetic materials such as Dacron and Gore-Tex have been used successfully as large-diameter (> 5mm) vascular grafts, resulting in good, long-term patency. However, because of the low flow conditions of small-diameter (< 5 mm) vessels, synthetic small-diameter vascular grafts accumulate plasma proteins and platelets quickly and occlude rapidly as shown in Figure 1-1. Currently, the preferred small-diameter vessel replacements are autogenous vessels from the patients’ saphenous veins or mammary arteries. However, the saphenous veins of many patients are unsuitable for use in these procedures or they have been previously harvested for other procedures. Edwards et al. report that this occurs in as many as 30% of patients, and Panetta et al. report that about 12% of the veins considered for arterial replacement were diseased, resulting in a more rapid re-occlusion [1.2, 3]. Even if a vascular replacement is successful, the patient is placed on a long-term regimen of inconvenient anticoagulant drug therapy with problematic side effects. Also, there are complications with using
Figure 1-1. Failed canine ePTFE vascular graft (cross-section). The white is the graft material and the dark red area is a thrombus that has completely occluded the graft.
veins, which have a different anatomy than that of arteries, to perform an arterial physiological role under increased flow and pressure conditions [1.4]. There is clearly a need for successful, long-term, synthetic, small-diameter, vascular grafts.

Graft materials have been sought after for almost a century, starting with Carrell, who in 1910 used heterografts that had been fixed in formalin for aortic replacement [1.5]. Later, many others also used vein and artery autografts, homografts, and heterografts [1.6-8]. In 1952, Voorhees et al. was the first to attempt arterial replacement with synthetic materials consisting of Vinyon “N” cloth [1.9]. Since then, there have been many attempts to improve the biocompatibility and long-term patency of vascular replacements. These attempts employ three main strategies: 1) Finding or creating a new material, synthetic or natural; 2) Altering the surface properties of a currently used material; or 3) Creating tissue-engineered vessels.

Many materials have been evaluated as grafts including, but not limited to Dacron (poly(ethylene terephthalate), PET), GoreTex (expanded poly(tetrafluoroethylene), ePTFE), polyethylene (PE), nylon, polydimethylsiloxane (PDMS), and polyurethane [1.10, 11]. In the best-case scenario, a Dacron or GoreTex graft in a large-diameter application, there is an 85-95% 5-year patency rate [1.12]. However, in a small diameter application, the 1-year patency rate is approximately 43%, and the 3-year rate around 30%, a less than ideal result [1.13].

Numerous attempts have been made to modify the previously mentioned materials in an effort to improve their interfacial properties with blood. The rationale is to use a material that already possesses desirable bulk properties such as strength, durability, and compliance and modify its surface to reduce its thrombogenicity. A carbon coating has
been shown to decrease surface thrombosis; however, this surface modification did not increase the patency of an ePTFE graft [1.14, 15]. Radio frequency glow discharge (RFGD) is an effective and easy way to modify material surfaces, and consequently has been used extensively to alter vascular grafts. A monomer can also be added during RFGD process, thus creating a surface polymer layer. A Dacron graft was treated with RFGD in the presence of tetrafluoroethylene monomer, creating a PTFE surface. This graft showed less thrombus formation when compared to Dacron or ePTFE grafts [1.16, 17]. N-vinyl-pyrrolidone and γ-butyrolactone are other common monomers used to modify, via RFGD, surfaces allowing them to resist thrombus formation and exhibit improved cellular growth [1.18]. Polyethylene glycol (PEG) can also be an effective surface modification to resist protein adsorption and ultimately thrombus formation [1.19]. The rationale is that for a protein to adsorb onto the surface, the well-hydrated surface PEG layer would have to dehydrate, an unlikely event resulting in a large entropic energy cost. Polyurethane surfaces may also be modified. In one example, a polyurethane surface was derivatized with 2-acrylamido-2-methylpropanesulphonic acid in order to imitate the charge and structure, and hopefully the anti-coagulant nature of heparin [1.20]. A Dacron graft was coated on the inside with polyurethane (for smoothness), and again coated with a 2-hydroxyethyl methacrylate and styrene copolymer (for nonthrombogenicity). These grafts showed no thrombus and no pannus ingrowth after one year in canines [1.21].

In addition to creating chemical surface modifications, grafts have also been modified with biological molecules selected for their passivity, antithrombogenic activity, or cell adhesive properties. Albumin, the most abundant blood protein, is often
considered for its passivity. Dacron grafts have been treated with sodium hydroxide creating surface carboxyl groups to which albumin was attached resulting in improved biocompatibility [1.22]. Collagen, gelatin, elastin, and fibrin have also been incorporated into graft surfaces to improve biocompatibility and non-thrombogenicity; however, long term graft performance was not improved [1.23-29]. Biologically active molecules that prevent thrombogenesis or promote anticoagulation have been incorporated into the inner graft surface. These molecules include heparin, urokinase, prostaglandin E, and hirudin, [1.30-33]. These modifications have had limited success and the long-term effects of these biologically active modifications is unknown. In an effort to promote cell adhesion onto grafts, collagen and fibronectin have been incorporated into grafts [1.34, 35]. However, it is difficult to encourage human endothelial cell migration and growth on a vascular graft in vivo.

A third approach for improving grafts is to incorporate engineered tissues into a graft or to create a completely tissue-engineered vessel [1.36]. The simplest method is to incorporate a layer of endothelial cells on the inside of a graft as a tissue engineered surface modification. The confluency of the endothelial cells (ECs) must be near 100% so no thrombogenic surface is exposed. However, simply seeding autologous ECs, especially in humans, results in a low cell density because the surface lacks the ligands for EC receptors [1.37]. There have been many attempts to create a suitable modification that would promote EC attachment and growth on graft surfaces. These strategies will be further investigated in Section 1.3 as material endothelialization is a goal of this thesis. There are two strategies to creating a tissue-engineered vessel: 1) Grow a vessel in vitro prior to implantation or 2) Implant a bioresorbable graft seeded with ECs, smooth muscle
cells, and fibroblasts that will degrade over time while the cells “grow” a natural vessel. Smooth muscle and endothelial cells are seeded onto a scaffold and are allowed to grow in a bioreactor, which provides optimal conditions for growth while conditioning the vessel for the pulsatile flow and pressures in the body. Again, concerns with these types of grafts are their weak mechanical strength compared to the Dacron and Gore-Tex materials and an EC confluency less than 100%, which could be exposing a thrombogenic surface to the blood [1.38-41]. The mechanical strength could be increased by placing a permanent polymer scaffold within the graft [1.42]. *In vivo* vessel tissue engineering via cell-seeded scaffolds also suffers from mechanical strength issues. The scaffold degrades before tissue of sufficient strength can be grown, resulting in aneurysmal dilation and rupture; however if the scaffold does not degrade fast enough, tissue will not perfuse into the scaffold [1.43-46]. One solution may be to use two scaffolds that degrade at different rates [1.47].

Despite all of the previously mentioned strategies for developing a vascular prosthesis, the classic problems of calcification [1.48, 49], bacterial infection [1.47, 50, 51], anastomotic hyperplasia, and thrombosis [1.52], which is initiated by protein adsorption [1.53, 54] and platelet deposition [1.55, 56], still remain. However, the most significant of the problems, being the cause of most graft failures, is the formation of thrombus, which consists mostly of fibrin and platelets [1.47].

Thrombosis on a vascular prosthesis is the result of two complementary mechanisms in the coagulation cascade: the intrinsic and extrinsic pathways. The extrinsic pathway occurs as a result of the surgical procedure, where the endothelium surrounding the prosthesis is damaged. In response to the tissue injury vascular wall cells
produce many factors and enzymes, including tissue thromboplastin (also called tissue factor or TF), that function to restore hemostasis and stimulate repair to the endothelium [1.57]. Then in the presence of Ca$^{++}$, Factor VII auto-activates to Factor VIIa (activated Factor VII), which in a TF / Factor VIIa complex cleaves Factor X to make Factor Xa [1.58]. Factor Xa, in complex with Ca$^{++}$ and Factor Va on the cell (EC or platelet) membrane (called the prothrombinase complex) converts prothrombin to thrombin.

Thrombin is then activated and free to cleave the fibrinopeptides from fibrinogen forming the fibrin monomer [1.59]. The fibrin monomer now has exposed positive charges in its central domain, which associate with the negatively charged D domains of other fibrin monomers, forming a half staggered array. This array is then chemically cross linked by Factor XIIIa to form the fibrin clot [1.60]. The fibrin clot, through its RGD tripeptide motif, is free to bind the GIIbIIIa receptor of activated platelets, which act to amplify thrombosis [1.61].

The intrinsic pathway of coagulation is initiated by contact activation when blood is exposed to foreign surfaces, either collagen at the site of injury or the polymer of the vascular prosthesis. The cascade begins by adsorption of Factor XII onto the surface and activation to form Factor XIIa. Factor XII can be activated in many ways [1.62]: autoactivation, especially on negatively charged surfaces, activation by Factor XIIa, or most importantly, activation by kallekrein or high molecular weight kininogen. Factor XIIa then converts Factor XI to Factor XIa, which then converts Factor IX to Factor IXa [1.63, 64]. Then on the cell (EC or platelet) membrane Factor IXa, along with Ca$^{++}$ and Factor VIIIa, forms the tenase complex, which acts to cleave Factor X to form Factor Xa [1.65]. Factor Xa then completes coagulation as discussed previously.
Platelets, as previously mentioned, are within the fibrin clot, but they are rapidly attracted to the site of tissue injury by binding to fibrinogen, fibronectin, vitronectin, and von Willebrand Factor (vWF) [1.66, 67]. Platelets can also adhere to foreign material by binding adsorbed proteins, such as fibrinogen or vWF. Platelets are activated at the site of thrombosis by agonists such as thrombin, epinephrine, serotonin, thromboxane A₂, and adenosine-5’-diphosphate [1.68, 69]. Once activated, platelets release coagulation factors and agonists such as ADP and serotonin, further amplifying the coagulation response.

1.2. Maintenance of Hemostasis by Vascular Endothelium

Since current small-diameter vascular grafts do not match the thrombo-resistance of a native vessel, it is important to investigate the endothelium and why it is so antithrombogenic. ECs have both passive and active (biochemical) mechanisms by which they resist thrombosis. On the surface of ECs are molecules that resist interaction with the blood components (coagulation factors, adhesion proteins, platelets, and cells) that constantly come into contact with the blood. Together, these molecules; mostly glycoproteins, glucosaminoglycans, and polysaccharides; are called the cell glycocalyx [1.70-75]. (See Figure 1-2.) Being highly hydrated, these molecules resist protein adsorption and cell attachment because adsorption and attachment would require dehydration of the glycocalyx—a significant entropic cost. In addition, due to the net negative charge of the glycocalyx, ECs may resist adhesion to platelets, which are also negatively charged. And although the EC surface is hydroxyl rich, making it a target for
Figure 1-2. TEM image of the endothelial cell glycocalyx. Reprinted from Bruce Alberts et al., Molecular Biology of the Cell. 3rd ed. Garland Pub., Inc., p. 502, Fig. 10-40, 1994.
the complement components C3 and C5, decay-accelerating factor (an EC surface
glycoprotein) makes ECs resistant to complement attack [1.76]. However, through cell
surface receptors, ECs still bind blood components; therefore, the glycocalyx alone is not
sufficient for thromboresistance.

The EC surface also contains molecules that actively inhibit the coagulation
cascade as shown in Figure 1-3. If thrombin binds and forms a complex with
thrombomodulin, an EC surface glycoprotein, protein C activation occurs [1.77].
Activated protein C in complex with protein S then inactivates Factors Va and VIIIa,
making Factors Vi and VIIIi [1.78-80]. ECs also release prostacyclin, also called
prostaglandin I2 (PGI2), a potent platelet aggregation inhibitor [1.81]. In addition, ECs
regulate the fibrinolytic system by secreting tissue plasminogen activator (tPA) and
urokinase plasminogen activator (uPA) that convert plasminogen to plasmin, which is
responsible for breaking up the fibrin clot [1.82, 83]. ECs also contain a cell surface
polysaccharide called heparan sulfate within proteoglycans called heparan sulfate
proteoglycans [1.84]. Heparan sulfate can form a complex with antithrombin III (ATIII),
which increases ATIII’s affinity for thrombin. Then the thrombin/ATIII complex can be
formed, which is an irreversible binding that permanently keeps thrombin from cleaving
fibrinogen [1.85]. ECs also act to degrade the platelet agonist, ADP via an ADPase. As
a result of the glycocalyx and these biochemical processes, hemostasis is carefully
maintained, until the endothelium is disrupted when ECs change to a procoagulant state.
Figure 1-3. Model of antithrombogenic properties of the endothelial cell. Antithrombin III (ATIII) with heparan sulfate proteoglycan (HSPG), inhibits coagulation enzymes producing protease/inhibitor complexes (TAT). Thrombomodulin (TM) binds thrombin (T) and catalyzes the activation of protein C (APC). APC with protein S (S) degrades factors Va and VIIIa. Tissue factor pathway inhibitor (TFPI) inhibits the tissue factor (TF) factor Xa complex. Tissue plasminogen activator (TPA) cleaves plasminogen to plasmin, which can degrade fibrin. EC release prostacyclin (PGI₂) and nitrous oxide (NO), which inhibit platelet activation. EC adhere to the extracellular matrix via integrin receptors and HSPG. Through focal adhesions (FA) the integrins can interact intracellularly with the actin cytoskeleton linking inside and outside of the cell. (Sagnella, Sharon M, Endothelial Cell Adhesion, Growth, and Function on Biomimetic Surfactant Polymers. 2003, Case Western Reserve University: Cleveland, Ohio)
1.3. Present Techniques in the Endothelialization of Biomaterials

As mentioned in the previous section, no man-made surface is as thromboresistant as unperturbed endothelium; therefore, since endothelial cells are nature’s hemocompatible surface, it would be ideal to have ECs on the surface of a vascular graft. However, it would be important that these ECs be in an anticoagulant state, because through the production of TF and other coagulation factors, as mentioned in section 1.1, disrupted ECs can be a powerful coagulation initiator.

There have been many attempts to endothelialize the inner surface of a graft [1.86]. First attempted in 1978, Herring et al. seeded ECs onto a Dacron graft (6 mm) and implanted the grafts into canine aortae [1.87]. Over time the endothelialized grafts showed significantly greater mean thrombus-free area when compared to Dacron alone. Later, Graham et al. successfully used endothelialized Teflon grafts in a canine model [1.88], and Meinhart et al. conducted a study of endothelialized ePTFE grafts in the infrainguinal position [1.89]. Using animal models, other groups have used endothelialization to increase the success of vascular grafts [1.90-94]. Unfortunately, the same degree of success has not been observed in humans.

Human ECs have a low affinity for current graft materials and many ECs are lost due to the shear forces experienced under conditions of blood flow [1.95-98]. EC retention has been shown to increase when the endothelialized graft is preconditioned under shear stress conditions [1.99]. To combat this EC loss effect, many known cell adhesive substances such as collagen [1.100], laminin [1.101, 102], fibrin [1.103], and fibronectin [1.104-107] have been used to precoat grafts and increase EC attachment.
However, ECs may still be lost to shear due to the weak bond between the adhesive proteins and the substrate material, exposing the underlying thrombogenic surface. Confluency is the most important factor for the success of endothelial cell seeded vascular grafts [1.37, 108].

Many materials have been developed that contain cell adhesive molecules. One of the most used is the RGD tripeptide, which is found in many matrix proteins. This peptide has been incorporated into many materials including Dacron [1.109, 110], Teflon [1.109], poly(vinyl alcohol) [1.111], polyacrylamide [1.112], poly(carbonate-urethane) [1.113], polyurethane [1.114, 115], PEG [1.116], and poly(lactic acid) [1.117]. However, the RGD tripeptide can also attach platelets, making these surfaces, if exposed, highly thrombogenic. YIGSR (from laminin) and REDV (from fibronectin) are other cell adhesive peptides found in matrix proteins that have been incorporated into materials [1.109, 118, 119]. It is interesting to note that the surface modified with REDV was EC specific and did not support the adhesion of fibroblasts, smooth muscle cells, and platelets. These adhesive peptides have been used in materials that also contain PEG to induce specific cell attachment and inhibit other cell attachment and protein adsorption [1.120-122]. Materials that contain growth factors, such as basic fibroblast growth factor, have also been created and been shown to enhance EC proliferation [1.123]. RFGD polymerization has been used to synthesize surfaces with increased nitrogen and/or oxygen content, which have been shown to increase EC attachment [1.124, 125].
1.4. Endothelium Attachment to the Extracellular Matrix

In order to design a material that encourages endothelialization, an understanding of the EC, its extracellular matrix (ECM), and their interaction must be possessed. The ECM is a mesh of glycoproteins and proteoglycans to which ECs adhere. ECs have glycoprotein adhesion receptors on the cell surface that serve for EC-EC adhesion and EC-ECM adhesion [1.126]. Cadherins, selectins, and cell adhesion molecules (CAM) are families of proteins for EC-EC adhesion. Some EC transmembrane proteoglycans can also be receptors for the ECM. The integrins, however, are primarily responsible for EC-ECM adhesion and are of particular interest with respect to material endothelialization.

In other words, for an EC to be strongly adhered, attachment must be via these structures. The integrins are transmembrane proteins that, when activated, can bind to the ECM and then attach to the actin cytoskeleton [1.127]. In order to attach, integrins must first become activated through “inside-out” signaling mechanisms [1.128], then once an attachment is made, it can be communicated through “outside-in” signaling mechanisms [1.129]. Integrins consist of an α and a β subunit. There are 15 known α subunits and 8 known β subunits that are known to occur in 21 different αβ dimers. Each integrin has a specific ligand to which it will attach, and these peptide motifs can be found in one or more ECM proteins. With respect to ECs, the α5β1 and the αvβ3 integrins are particularly important, both of which bind to a derivative of the RGD motif [1.130]. The α5β1 integrin is the one that is thought to be more responsible for establishing tight attachments with the surface and the αvβ3 integrin plays a larger role in cell migration, angiogenesis, wound healing, and tumor metastasis [1.131, 132]. These two integrins
also have different signaling mechanisms [1.133]. Table 1-1 lists integrins present in ECs, their ligands, and the particular peptide motif in the ligand responsible for the interaction.

<table>
<thead>
<tr>
<th>Adhesion Molecules</th>
<th>Ligand or Counter-Receptors</th>
<th>Peptide sequence in ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin Superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α2β1</td>
<td>COL, LM</td>
<td></td>
</tr>
<tr>
<td>α3β1</td>
<td>FN, COL, LM</td>
<td></td>
</tr>
<tr>
<td>α4β1</td>
<td>FN</td>
<td>REDV, LDV</td>
</tr>
<tr>
<td>α5β1</td>
<td>FN</td>
<td>RGDS</td>
</tr>
<tr>
<td>α6β1</td>
<td>FN, LM</td>
<td></td>
</tr>
<tr>
<td>αvβ3</td>
<td>VN, vWF, FG, FN</td>
<td>RGDV</td>
</tr>
<tr>
<td>αvβ5</td>
<td>VN, vWF</td>
<td></td>
</tr>
<tr>
<td>Ig Gene Superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>αLβ2, αMβ2</td>
<td></td>
</tr>
<tr>
<td>ICAM-2</td>
<td>αLβ2</td>
<td></td>
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<tr>
<td>PECAM-1</td>
<td>?</td>
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<tr>
<td>VCAM-1</td>
<td>α4β1</td>
<td></td>
</tr>
<tr>
<td>Selectins</td>
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<tr>
<td>E-Selectin</td>
<td>CLA</td>
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<tr>
<td>P-Selectin</td>
<td>Sialyl Lewis X</td>
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<tr>
<td>Leucine-Rich Motif Family</td>
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<tr>
<td>GP 1b</td>
<td>VWF</td>
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<tr>
<td>Cadherins</td>
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<tr>
<td>V-Cadherin</td>
<td>V-Cadherin</td>
<td></td>
</tr>
<tr>
<td>“Orphan” CAMs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP IV</td>
<td>TSP</td>
<td></td>
</tr>
</tbody>
</table>

Modified from [1.126] and [1.130]
FN, fibronectin; VN, vitronectin; vWF, von Willebrand factor; TSP, thrombospondin; LM, laminin; FG, fibrinogen; COL, collagen

31
ECM binding through EC surface proteoglycans that contain heparan sulfate or chondroitin sulfate is less specific and involves ECM proteins that contain heparin-binding domains [1.134]. The peptide sequences in these domains consist of clusters of basic residues spaced apart by hydrophobic residues, such as RRAR and PRPRP [1.135-140]. It is proposed that these heparin-binding interactions reinforce the integrin adhesion complexes [1.141, 142]. Laminin also has a notable peptide sequence, YIGSR that interacts with a cell laminin receptor, which is neither an integrin nor a proteoglycan [1.143].

The ECM is a supra-molecular network composed of many different biological macromolecules including collagens, fibronectin, thrombospondin, entactin, laminin, and proteoglycans [1.144, 145]. The ECM is a dynamic structure where ECs can affect it in various ways; however, the makeup of the ECM can also have a profound effect upon ECs [1.146-149]. These ECM components are large, complex, and multi-functional; they contain sites for binding each other and EC surface receptors as well as serving structural roles.

1.5. Fibronectin—Structure and Endothelial Cell Attachment Function

Although ECs adhere to many components of the ECM that may be utilized for encouraging endothelialization, fibronectin is regarded as the key constituent and has been the preferred adhesion molecule [1.150-156]. Therefore, to create a surface hospitable to ECs, the structure and cell attachment function of fibronectin must be
understood. Fibronectin (FN) has an approximate molecular weight of ~250,000 and is a dimeric glycoprotein where the two subunits are disulfide bonded at the C-termini [1.157, 158]. FN in the ECM exists as a fibrillar network [1.159, 160]. FN contains three different types of conserved domains named FN-I, FN-II, and FN-III (Figure 1-4A). Typically, there are nine FN-I and two FN-II domains at the N-terminus, followed by 15 FN-III domains. At the C-terminus there are an additional three FN-I domains. FN-I consists of two different antiparallel β sheets (three strands in one sheet and two strands in the other). FN-II also consists of two antiparallel β sheets. FN-III is a β sandwich structure—one antiparallel β sheet (consisting of 3 strands) right next to another antiparallel β sheet (consisting of 4 strands).

The domains of FN are highly functional, corresponding to binding regions for adhesion molecules and ECM proteins [1.161]. Figure 1-4B shows the distinct binding regions in FN, which were found by binding the products of FN enzymatic digestion or site directed mutagenesis to different proteins [1.162, 163]. Of particular interest are the cell-binding region for the α5β1 integrin (FN-III8-10) and one of the heparin binding regions (FN-III12-14). The cell-binding region contains the RGDS peptide motif in FN-III10 and a synergy sequence (PHSRN) in FN-III9 that aids in binding to α5β1 [1.164-166]. The heparin-binding region of FN (shown in Figure 1-5) has been attributed to the WQPRRARI, SPPRRARVT, and PRRARVTADETTITISWRTKT peptide sequences [1.167, 168]. However, there are also other basic residues within FNIII12-14 that aid in heparin binding [1.169]. It has been hypothesized that for cells, binding to the heparin-binding region can supplement integrin binding [1.142]. Binding to the heparin-binding region may be necessary for forming focal adhesions with the α5β1 integrin [1.170].
Figure 1-5. Heparin binding region of FN. Figure adapted from Sharma, A., et al., Crystal structure of a heparin- and integrin-binding segment of human fibronectin. Embo J., 1999. 18(6): p. 1468-79.
1.6. Design of Amphiphilic Polymer Surfactants for EC Attachment

Since human ECs are incompatible with and will not attach successfully to current hydrophobic biomaterials, an interface must be developed that will adhere to a polymer surface while facilitating EC attachment and proliferation. A design is proposed based upon a previously synthesized polymer surfactant consisting of a poly(vinyl amine) (PVAm) backbone and two sets of ligands [1.171]. The use of a polymer surfactant as a surface modification is quite effective and can be relatively easy to carry out by a simple dip coating process. Here, for this research, the ligands will be tailored for polymer adherence and extracellular matrix biomimicry as shown in Figure 1-6. The hydrophobic alkyl ligands are for adsorption onto the hydrophobic surface. There are two peptide ligands for the promotion of EC attachment. One peptide’s sequence is GSSGGRGDSPA. The GRGDSPA amino acid sequence, derived from the cell-binding region of FN, is designed for interaction with the $\alpha_5\beta_1$ integrin of an EC, while the GSSS is a hydrophilic spacer sequence design to elevate the functional sequence of peptide into the solution to enable its interaction with the integrin. The other peptide also consists of a hydrophilic GSSSG spacer and a functional sequence derived from fibronectin. However, this second peptide ligand is designed as a heparin binding peptide (HBP), which will facilitate EC interaction via the cell surface heparan sulfate proteoglycans. It has been suggested that for complete binding via $\alpha_5\beta_1$, interactions with cell surface proteoglycans are also necessary [1.172]. Three possible HBPs, whose functional sequences have been shown to bind heparin, are proposed here: 1-GSSSGWQPRARI (HBP1), 2-GWSSGSPRRARVT (HBP2), and 3-
Figure 1-6. Model of extracellular matrix-like surfactant polymer. The polymer consists of a PVAm backbone to which many different ligands may be attached: Alkyl side chains for adsorption onto the substrate material, well-hydrated sugars to resist protein adsorption, integrin binding peptides for cell attachment, and heparin binding peptides to promote cell adhesion.
GSSSGSPPRRARVTDATETTITISWRTKT (HBP3). Other peptide sequences, such as cyclic RGD peptides or peptides from the synergy region of FN, can also be introduced into the design to further facilitate integrin binding. Finally, a third small hydrophilic ligand, possibly a small sugar like maltose, can be reacted at the remaining backbone amines. This sugar would complete a well-hydrated surface layer to resist any protein adsorption while not interfering with the peptides and their interactions. Also, the design of the surfactant polymer allows for the tailoring of the ligand ratios to meet the needs of a specific application and optimize the properties.

1.7. Research Objectives

Before the complete ECM-like surfactant polymer in the previous section can be implemented, the individual hydrophilic ligands and their interactions must be studied to determine the contribution of each to the ECM-like surfactant polymer. It is the purpose of this thesis to characterize surfactant polymers that contain alkyl ligands along with RGD peptides, RGD peptides with dextran sugars, HBP1, HBP2, HBP1 with RGD peptides, and HBP2 with RGD peptides. The hypothesis is that surfactant polymers with these peptide ligands adsorbed onto the surface will provide an interface that will promote EC attachment and growth.

The multi step syntheses and purifications of PVAm(RGD:Hex) and PVAm(RGD:Dex:Hex) will be discussed in Chapter 3. The multi step syntheses and purifications of PVAm(HBP1:Hex), PVAm(HBP2:Hex), PVAm(HBP1:RGD:Hex), and
PVAm(HBP2:RGD:Hex) will be discussed in Chapter 4. In both chapters, the synthesis will be characterized at each step via mass spectrometry, infrared spectroscopy, nuclear magnetic resonance spectroscopy (NMR), and high-pressure liquid chromatography. Insights into the surfactant polymers’ ligand compositions will be drawn from molecular modeling studies of the peptides and from $^1$H-NMR integral ratios. The surfactant polymers will be adsorbed onto octadecyltrichlorosilane treated glass—a model hydrophobic substrate. The surfactant modified surfaces will then be characterized by contact angle goniometry, x-ray photoelectron spectroscopy, and atomic force microscopy to evaluate the quality of the coating. Finally, the bioactivity of the modified surfaces will be determined with the seeding of ECs and with the aid of phase contrast and laser scanning confocal microscopies. The proof-of-concept synthesis and characterization of a peptide ligand containing both the synergy peptide and the integrin-binding RGD peptide will be discussed in Chapter 5. In the future this synergistic ligand can be incorporated into a surfactant polymer. Chapter 6 details the synthesis and characterization of a cyclic RGD ligand for incorporation into a surfactant polymer, and Chapter 7 shows additional synthesis, characterization, and modeling experiments relating to peptide surfactant polymers. Conclusions will be drawn for the further development of ECM-like polymer surfactants possibly leading to completely endothelialized, and therefore, thromboresistant cardiovascular biomaterials.
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Chapter 2. SYNTHETIC STRATEGIES OF BIOMIMETIC PEPTIDES FOR INCORPORATION INTO BIOMATERIALS

The objective of much of this thesis is to synthesize biomimetic peptides with a distinct functional group or one unprotected functional group for bioconjugation to a material resulting in a single selective configuration for the immobilized peptide. This chapter will introduce the method of solid phase peptide synthesis with the goal of exploiting a singular and specific functional group on the peptide for conjugation onto a biomaterial/biomaterial modification.

2.1. Merrifield

By the 1950’s short peptides were successfully being synthesized; however, the process was time consuming, labor intensive, and resulted in low yields of the product. Bruce Merrifield had been synthesizing peptides up to seven amino acids in length by conventional methods, when “on May 26th, 1959 he wrote in his laboratory notebook ‘There is a need for a rapid, quantitative, automatic method for the synthesis of long chain peptides.’” [2.1] He then outlined an elegant and revolutionary new strategy for the synthesis of peptides, and on July 20th, 1963, “Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide” was published in the Journal of the American Chemical Society [2.2]. He used polystyrene beads 20-80 µm in diameter crosslinked with 2% divinylbenzene, which is still the industry standard today, as a solid support for the
growing peptide chain. Then, following each successive reaction step of coupling an amino acid and deprotecting the amino acid terminus, reagents and unused reactants could be washed away by simple filtration, thus preserving the product, enhancing the yield, and eliminating the need for purification by recrystallization at every step. Using his solid phase method, reactants could be used in excess to drive the reaction further to completion, also greatly enhancing product yield.

Specifically, his method is outlined in Scheme 2-1 [2.2]. First an esterification reaction was performed by refluxing a chloromethylated polystyrene resin and a carbobenzoxy-protected amino acid (Scheme 2-1A). Next, the carboenzoxy protecting group was removed from the amino terminus of the amino acid using hydrobromic acid and acetic acid (Scheme 2-1B). Then, using a carbodiimide, a peptide bond is formed with the next carboenzoxy-protected amino acid (Scheme 2-1C). Steps B and C in Scheme 2-1 can be successively repeated to propagate the peptide chain; these alternating reactions are the heart and soul of solid phase peptide synthesis (SPPS). Finally, the carboenzoxy protecting group was removed from the amino terminus of the peptide using hydrobromic acid and acetic acid and a sodium hydroxide solution was used to cleave the peptide from the polystyrene resin (Scheme 2-1D).

He later automated the solid phase peptide synthetic process and could make peptides 20 times faster compared to solution methods. Bruce Merrifield was awarded the Nobel Prize in Chemistry in 1984 for this work. He recently died within this past year on May 14, 2006.
2.2. Principle of Orthogonality

The method of solid phase peptide synthesis is predicated on the Principle of Orthogonality. Two protecting groups, X and Y, are orthogonal if the conditions for removal of one group from the peptide have no effect on the stability of the other. X can be removed with the Y protecting groups remaining, and Y can be removed with X remaining. An example is where X is removed by basic conditions while acidic conditions remove Y.

The nuance of pseudo-orthogonality is also often exploited in solid phase peptide synthesis. Two protecting groups, X and Y, are pseudo-orthogonal if the removal of one group from the peptide has a small effect on the stability of the other while the removal of the other group ends up removing both groups. X can be removed with most of the Y protecting groups remaining; however, the removal of Y will also remove all of the X protecting groups. An example would be where X is removed by mildly acidic conditions while strongly acidic conditions remove Y.

2.3. Chemistries of Peptide Synthesis

2.3.1. Boc Chemistry

Merrifield’s laboratory and other laboratories continued to improve the chemistries used in the solid phase peptide synthetic method. Two common chemistries
used today are Boc chemistry and Fmoc chemistry. These chemistries are named for the protecting groups on the propagating amino terminus of the peptide. Boc (t-butyloxycarbonyl) replaces carbobenzoxy N-α-amino protecting group on the amino acid that Merrifield used. The Boc group is usually removed under acidic conditions with 50% (v/v) trifluoroacetic acid solutions. The bonds between the amino acid side chains and their protecting groups and between the peptide carboxylate terminus and the solid support resin are broken under strong acidic conditions using either hydrofluoric acid (HF) [2.3, 4], trifluoromethanesulfonic acid (TFMSA) [2.5], or trimethylsilyloxytrifluoromethanesulfonate (TMSOTf) [2.6]. The Boc chemistry technique is pseudo-orthogonal, because with each Boc deprotection up to 0.7% of the peptide can be cleaved from a Merrifield type resin and 0.007% of the peptide can be lost with a 4-(oxymethyl)-phenylacetamidomethyl (PAM) resin [2.7].

2.3.2. Fmoc Chemistry

Fmoc chemistry is the current industry standard for the chemistry of solid phase peptide synthesis. Fmoc (9-fluorenylmethyloxycarbonyl) replaces carbobenzoxy N-α-amino protecting group on the amino acid that Merrifield used. The Fmoc group is removed under basic conditions with a 20% (v/v) piperidine solution [2.8, 9]. The bonds between the amino acid side chains and their protecting groups and between the peptide carboxylate terminus and the solid support resin are broken under acidic conditions using
either trifluoroacetic acid [2.10] or trimethylsilyl bromide [2.6]. The Fmoc chemistry technique is orthogonal.

2.4. Coupling

Methods for coupling each successive amino acid onto the peptide chain by forming an amide bond are similar. They usually involve a reagent that forms an activated complex with the α-carboxylate of the amino acid, making the α-carbonyl carbon more electrophilic and more susceptible to nucleophilic attack by the exposed N-terminal amine of the peptide chain. Some of these carboxylate activating reagents are N,N'-dicyclohexylcarbodiimide (DCC) [2.11], other carbodiimides [2.12-15], benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) [2.16], N,N-bis[2-oxo-3-oxazolidinyl]phosphorodiamidic chloride (BOP-Cl) [2.17], Bromo-tris-pyrrolidino phosphoniumhexafluorophosphate (PyBroP) [2.18], bromotris(dimethylamino)phosphonium hexafluorophosphate (BrOP) [2.19, 20], PyBOP [2.21], O-(Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) [2.22], 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) [2.23], and O-(7-Azabenzo triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) [2.24]. The most commonly used are HATU, HBTU, and DCC. Since the activated ester formed between the α-carboxylate and the activating reagent is short lived, sometimes a second reagent like N-hydroxysuccinimide [2.25], 1-hydroxy-7-azabenzo triazole (HOAt) [2.24], or 1-hydroxybenzotriazole (HOBt) [2.26] is
used that forms a longer lived entity, thus increasing the amount of time available for and the chances of reacting with the peptide’s amino terminus. Sometimes preformed activated esters are used like the pentafluorophenyl ester [2.27] and the 3-hydroxy-2,3-dihydro-4-oxo-benzo-triazene ester [2.28]. N-carboxyanhydrides and symmetrical anhydrides of the amino acids have also been used for coupling [2.29, 30]. The most common coupling methods currently used are HBTU/HOBt, HATU/HOAt, or DCC/HOBt.

2.5. Side-chain Protecting Groups

Merrifield’s first synthesis, Leu-Ala-Gly-Val, benefited from the fact that all of the amino acid side chains in the peptide were aliphatic and did not possess any functional groups that would interfere with the amino acid coupling or the N-terminal deprotection reactions. However, a lot of amino acids have side chains with functional groups that could participate with the coupling reactions. The alcohols of serine, threonine, and tyrosine could couple with an amino acid to form a side chain ester. The carboxylates of aspartate and glutamate could couple with an amino acid to form a side chain anhydride. The amine of lysine could couple with an amino acid to form a side chain amide, and the thiol of cysteine could couple with an amino acid to form a side chain thioester. Asparagine, glutamine, arginine, histidine, and tryptophan can also participate in side reactions. In order to synthesize a peptide containing any of these amino acids, their side chains need to be protected. The side chain protecting groups
used in Fmoc peptide syntheses are shown in Table 2-1. The alcohols of serine, threonine, and tyrosine are typically protected as t-butyl ethers, which cannot react with an amino acid during the coupling step. The carboxylates of aspartate and glutamate are typically protected as t-butyl esters. The amine of lysine is typically protected as a t-butyl urethane, and the thiol of cysteine is typically protected as a tritylthioether. Commonly, the side chain protecting groups are labile in the same manner as the peptide-resin linkage, so that after the peptide synthesis is complete, the protecting groups are removed at the same time under the same conditions as cleavage from the resin.

In nature, a protein is rarely a simple linear string of amino acids. Proteins can have amino acids that are glycosylated, or be conjugated to a lipid, or have loops where cysteines are joined together as disulfide bridges. Recreating these moieties synthetically require an additional degree of synthetic difficulty. If, for example, one wanted to make a glycosylated peptide from human erythrocyte glycophorin residues 30-40 (Lys-Arg-Asp-Thr-Tyr-Ala-Ala-Thr-Pro-Arg-Ala), where only Thr$_{36}$ is glycosylated, and all the other amino acids are not, then standard t-butyl ether protecting groups would not allow one to selectively glycosylate the one threonine residue while not glycosylating any others. Therefore, there is a need for another degree of orthogonality in the side chain protecting groups. Table 2-1 shows that tritylether threonines are pseudo-orthogonal to t-butyl threonines, so one solution would be to synthesize the entire peptide using standard amino acid protection except for Thr$_{36}$, which could be protected as a tritylether. Then, while the peptide is still attached to the resin, the trityl group could be removed using 1% TFA. This exposes only Thr$_{36}$ for the bioconjugation of a sugar molecule. Finally, the glycopeptide could be deprotected and cleaved for the solid support using 85% TFA.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Protecting Group</th>
<th>Cleavage Conditions</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>Pbf</td>
<td>95% TFA</td>
<td>standard, more acid labile than Pmc</td>
</tr>
<tr>
<td></td>
<td>Pmc</td>
<td>95% TFA</td>
<td></td>
</tr>
<tr>
<td>Mtr</td>
<td></td>
<td>95% TFA 35°C</td>
<td>more acid stable than Pmc</td>
</tr>
<tr>
<td>Asn</td>
<td>Trt</td>
<td>95% TFA</td>
<td>standard more stable to acidolysis than Mtt</td>
</tr>
<tr>
<td>Gln</td>
<td>Resin</td>
<td>TFA or HF, etc.</td>
<td>depends on chosen resin</td>
</tr>
<tr>
<td></td>
<td>OtBu</td>
<td>95% TFA</td>
<td>standard</td>
</tr>
<tr>
<td></td>
<td>OPp</td>
<td>1% TFA</td>
<td>pseudo-orthogonal to OtBu, on-resin modification</td>
</tr>
<tr>
<td>Asp</td>
<td>OBzl</td>
<td>H₂/Pd or HF</td>
<td>pseudo-orthogonal to OtBu</td>
</tr>
<tr>
<td>Glu</td>
<td>OAll</td>
<td>Pd(PPh₃)₄</td>
<td>orthogonal to Fmoc/tBu/resin linkage</td>
</tr>
<tr>
<td></td>
<td>ODMab</td>
<td>2% N₂H₄H₂O</td>
<td>pseudo-orthogonal to Fmoc, acid stable</td>
</tr>
<tr>
<td></td>
<td>Resin</td>
<td>TFA, HF, or AcOH</td>
<td>depends on chosen resin</td>
</tr>
<tr>
<td></td>
<td>Trt</td>
<td>95:5 TFA:EDT</td>
<td>standard</td>
</tr>
<tr>
<td>Cys</td>
<td>Acm</td>
<td>I₂ in 80% AcOH (aq)</td>
<td>orthogonal</td>
</tr>
<tr>
<td></td>
<td>Mob</td>
<td>TFMSA/TFA/anisole</td>
<td>orthogonal</td>
</tr>
<tr>
<td>His</td>
<td>Trt</td>
<td>95% TFA</td>
<td>standard</td>
</tr>
<tr>
<td></td>
<td>DNP</td>
<td>thiophenol/TEA</td>
<td>orthogonal</td>
</tr>
<tr>
<td>Lys</td>
<td>Boc</td>
<td>95% TFA</td>
<td>standard</td>
</tr>
<tr>
<td></td>
<td>Aloc</td>
<td>Pd(PPh₃)₄</td>
<td>orthogonal to Fmoc/tBu/resin linkage</td>
</tr>
<tr>
<td></td>
<td>Adpoc</td>
<td>1% TFA</td>
<td>pseudo-orthogonal to Boc, on-resin modification</td>
</tr>
<tr>
<td></td>
<td>Mtt</td>
<td>1% TFA</td>
<td>pseudo-orthogonal to Boc, on-resin modification</td>
</tr>
<tr>
<td></td>
<td>Dde</td>
<td>2% N₂H₄H₂O</td>
<td>pseudo-orthogonal to Fmoc, acid stable</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>H₂/Pd or HF</td>
<td>pseudo-orthogonal to Boc</td>
</tr>
<tr>
<td></td>
<td>Fmoc</td>
<td>20% piperidine</td>
<td>branched peptides</td>
</tr>
<tr>
<td>Ser</td>
<td>tBu</td>
<td>95% TFA</td>
<td>standard</td>
</tr>
<tr>
<td>Thr</td>
<td>Trt</td>
<td>1% TFA</td>
<td>pseudo-orthogonal to OtBu, on-resin modification</td>
</tr>
<tr>
<td>Tyr</td>
<td>BzI</td>
<td>H₂/Pd or HF</td>
<td>pseudo-orthogonal to OtBu</td>
</tr>
</tbody>
</table>

**Table 2-1.** Amino acid protecting groups for Fmoc synthesis.
In the ultimate case of complexity, Table 2-1 shows that the capability exists to
make a peptide containing lysines with up to six different degrees of
orthogonality/pseudo-orthogonality. This means that up to six different reactions could
be selectively performed on up to six different lysines in a hypothetical peptide. The
Fmoc protecting group is deprotected using a 20% piperidine solution. The Aloc
(allyloxy carbonyl) protecting group is deprotected by reduction using Pd(PPh$_3$)$_4$ [2.31].
The Dde (1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl) protecting group is
deprotected using a 2% hydrazine solution [2.32]. The Mtt (4-methyltrityl) protecting
group is deprotected using a 1% TFA solution [2.33]. The Boc (t-butyloxycarbonyl)
protecting group is deprotected using a 85%-95% TFA solution [2.34], and the Z
(benzyloxycarbonyl) protecting group is deprotected using a hydrogenation [2.35],
hydrofluoric acid [2.3, 4], trifluoromethanesulfonic acid [2.5], or
trimethylsilyloxytrifluoromethanesulfonate [2.6].

Peptides with multiple disulfide bridges can be synthesized. Scheme 2-2
schematically shows the synthesis of a hypothetical peptide with multiple disulfide
bridges. First, the linear peptide sequence containing three different sets of cysteines,
protected with Trt (Trityl), Acm (Acetamidomethyl), and Mob (4-Methoxybenzyl), is
synthesized on the solid support resin using standard methods (Scheme 2-2A). Upon
cleavage from the resin the Trt groups are removed using 85% TFA (Scheme 2-2B), and
the first disulfide bridge is formed by oxidation in air (Scheme 2-2C). Next, the Acm
protecting groups are removed using iodine oxidation and the second disulfide forms as a
result of the deprotection (Scheme 2-2D) [2.36]. Finally, the Mob protecting groups are
Scheme 2-2. Synthesis of peptide with multiple disulfide bridges. ■ represents Trt protecting groups. ▲ represents Acm protecting groups. ● represents Mob protecting groups. “C” represents cysteine. Other amino acids are not shown for simplicity.
removed using the standard TFMSA method and the third disulfide in then formed by oxidation (Scheme 2-2E).

### 2.6. Resins

There are two main criteria to consider when choosing a solid support resin for solid phase peptide synthesis: 1) The conditions for cleavage of the peptide from the resin and 2) The functionality of the C-terminus of the peptide upon cleavage from the resin. Table 2-2 shows a selection of commercially available resins for solid phase peptide synthesis. The conditions of peptide cleavage for each resin are displayed in the third column. Most times, the cleavage conditions are chosen for convenience. For example, if performing a peptide synthesis using Fmoc-amino acids with t-butyl, Boc, Trt, and Pbf side chain protections, then peptide linkage with a PAL, Knorr, or Wang resin will be broken during the same reaction as side chain deprotection, thus eliminating the need for extra steps. Alternatively, if performing a peptide synthesis using Fmoc-amino acids with t-butyl, Boc, Trt, and Pbf side chain protections and using a 2-chlorotrityl chloride resin, then using very mild acidic conditions will cleave the peptide from the resin with side chain protection intact, which would allow the peptide be selectively conjugated to a lipid, polymer, scaffold, material, etc. without interfering side reactions with the amino acid side chains. Another interesting cleavage method is photolysis used with the brominated Wang resin or the Fmoc-photolabile resin, which can also provide fully protected peptides upon cleavage.
<table>
<thead>
<tr>
<th>Resin</th>
<th>Attachment Method</th>
<th>Cleavage</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang resin</td>
<td>DIC/HOBt/DMAP</td>
<td>50% TFA/DCM</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>Knorr resin</td>
<td>Remove Fmoc then</td>
<td>20% to 50% TFA/DCM</td>
<td>Peptide Amides</td>
</tr>
<tr>
<td>PAL resin</td>
<td>DIC/HOBt/DMAP</td>
<td>20% to 50% TFA/DCM</td>
<td>Peptide Amides</td>
</tr>
<tr>
<td>HMPB-BHA resin</td>
<td>DIC/HOBt/DMAP</td>
<td>1% to 2% TFA</td>
<td>Protected Peptide Acids</td>
</tr>
<tr>
<td>2-Chlorotrityl Chloride resin</td>
<td>Fmoc-amino acid plus DIPEA</td>
<td>1% TFA AcOH</td>
<td>Protected Peptide Acids</td>
</tr>
<tr>
<td>Knorr-2-Chlorotrityl resin</td>
<td>Remove Fmoc then</td>
<td>1% TFA AcOH</td>
<td>Protected Peptide Amides</td>
</tr>
<tr>
<td>Hydrazine-2-Chlorotrityl resin</td>
<td>DIC/HOBt</td>
<td>1% to 5% TFA/DCM</td>
<td>Protected Peptide Hydrazides</td>
</tr>
<tr>
<td>Fmoc-Photolable resin</td>
<td>Remove Fmoc then</td>
<td>Photolysis</td>
<td>Protected Peptide Amides</td>
</tr>
<tr>
<td>Phenol resin</td>
<td>DIC/HOBt/DMAP</td>
<td>NaOEt then NaOH</td>
<td>Protected Peptide Acids</td>
</tr>
<tr>
<td>BAL resin</td>
<td>Amino acid ester, NaBH(OAc)_3</td>
<td>same as previous resin</td>
<td>same as previous resin</td>
</tr>
<tr>
<td>Weinreb resin</td>
<td>DIC/HOBt</td>
<td>LiAIH4</td>
<td>Peptide Aldehydes</td>
</tr>
<tr>
<td>Glycerol resin</td>
<td>Fmoc-amino aldehyde, toluene sulfonic acid, drying agent</td>
<td>Aqueous acid</td>
<td>Peptide Aldehydes</td>
</tr>
<tr>
<td>Fmoc-Hydroxylamine 2-chlorotrityl resin</td>
<td>DIC/HOBt</td>
<td>1% to 2% TEA AcOH</td>
<td>Protected Peptide Hydroxamic acids</td>
</tr>
<tr>
<td>Merrifield resin</td>
<td>Cesium salt or KF</td>
<td>HF, TFSMA, or TFSOTf</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>PAM resin</td>
<td>DIC/HOBt/DMAP</td>
<td>HF</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>TentaGel S Br</td>
<td>Cesium salt or KF</td>
<td>NaOH/H_2O</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>TentaGel S OH</td>
<td>DIC/HOBt/DMAP</td>
<td>NaOH/H_2O</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>Chloroacetyl polystyrene</td>
<td>Cesium salt or KF</td>
<td>Tetrabutyl-ammonium fluoride, Trimethyltin hydroxide</td>
<td>Peptide Acids</td>
</tr>
<tr>
<td>Brominated Wang resin</td>
<td>Cesium salt or KF</td>
<td>Photolysis</td>
<td>Protected peptide acids</td>
</tr>
<tr>
<td>MBHA resin</td>
<td>Desalt then DIC/HOBt</td>
<td>HF, TFSMA, or TFSOTf</td>
<td>Peptide Amides</td>
</tr>
</tbody>
</table>

Table 2-2. A selection of resins that can be used for solid phase peptide synthesis. Commonly used resins are highlighted.
The functionality of the synthesized peptide’s C-terminus for a selection of commercially available resins for solid phase peptide synthesis is shown in the fourth column of Table 2-2. Most commonly the C-terminus is either an amide or an acid. An amide C-terminus is useful in preventing the C-terminus from participating in future peptide modifications on bioconjugation reactions. A carboxylic acid C-terminus can be useful for bioconjugation to a lipid, polymer, scaffold, material, etc. For synthesis using Boc chemistry, the PAM resin gives an acidic C-terminus, and the MBHA resin gives an amide terminus. For an Fmoc synthesis, the Knorr and PAL resin give an amide C-terminus, and the Wang and 2-chlorotrityl chloride resin give an acid terminus.

Another consideration is if the peptide synthesis will involve Boc chemistry or Fmoc chemistry. The peptide-resin bond on resins typically used for Fmoc synthesis like Knorr, PAL, Wang, and 2-chlorotrityl chloride is broken using TFA or milder acids. If any of these resins were used in Boc chemistry peptide synthesis, then the TFA N-terminal deprotection used with Boc chemistry would prematurely break the peptide-resin linkage. The PAM and MBHA resin are typically used with Boc syntheses; however, these resins could be used for Fmoc peptide synthesis as long as the stronger acidic conditions (HF, TFMSA, or TMSOTf) were used to break the peptide-resin linkage.

A final consideration is the length of the peptide chain. If the peptide has a very short length, it is more efficient to use a resin with a high density of reactive functional groups, also called the degree of substitution. Usually there is no detriment to the quality of the synthesis. If the peptide has a very long length, then the possibility exists that the peptide chains could start to interact with neighboring chains and then aggregate, which would hinder the synthesis. In this case, a resin with a low degree of substitution will
decrease the likelihood that neighboring chains will aggregate. An average degree of substitution of a peptide synthesis resin is 0.5-1.0 mmol/g.

2.7. Other Peptide Synthetic Strategies

2.7.1. Fluorescent labeling.

Sometimes it is required for future experiments to label a peptide with a fluorescent probe. The amino terminus and a lysine ε-amine are the most common functional groups for conjugating a fluorescent probe; especially when most probes are available as activated succimidyl esters or isothiocyanates, both which readily react with amines. Labeling the amino terminus can be easily achieved by reacting the fluorescent probe with the amino terminus while the peptide is still attached to the solid support resin. This is due to the fact that any ε-amines of lysines would still be protected and therefore not be labeled.

A situation could arise when it is desirable to label a particular lysine and not the amino terminus nor any other lysine in the peptide. This situation is depicted in Scheme 2-3. A fluorescent label is desired on the second lysine and not the first. First, the linear peptide is synthesized on a Knorr resin (Scheme 2-3A). The protected amino acid used for the second lysine is protected with a group that is orthogonal to the Boc group on the first lysine. This orthogonal group could be Aloc, which is deprotected with Pd(PPh₃)₄; Mtt, which is deprotected with 1%TFA; or Dde, which is deprotected with 2% hydrazine
Scheme 2-3. Fluorescent labeling of a specific lysine in a peptide. To avoid confusion only the lysine amino acids are shown and all other amino acids in the sequence are labeled “X.” The “■” shows protecting groups that are removed by 85% TFA. The “▲” shows an orthogonal protecting group. The “star” depicts the fluorescent probe.
(See Table 2-1). Next, the orthogonal group is removed (Scheme 2-3B). Then, the fluorescent succimidyl ester or isothiocyanate is reacted with the exposed \( \varepsilon \)-amine (Scheme 2-3C). Finally, the peptide is fully deprotected and removed from its solid support using 85% TFA (Scheme 2-3D).

### 2.7.2. Resin as a protecting group.

As is shown in Table 2-1, the solid support itself can serve as a side chain protecting group for aspartic acid or glutamic acid. These amino acids can be acquired with the side chain unprotected and the \( \alpha \)-carboxylate protected with either a benzyl ester, an allyl ester, or a t-butyl ester. The side chain acid can then be attached to the resin. A similar strategy was used by Tselios et al. [2.37]. The peptide Fmoc-glu-asn-pro-val-NH\(_2\) was synthesized and then cleaved from the resin. This peptide still has the N-terminus protected, and the C-terminus is capped with an amide functionality. This peptide was then loaded onto a 2-chlorotrityl chloride resin using the peptide’s only unprotected functional group—the \( \gamma \)-carboxylic acid. Peptide synthesis then followed to make Boc-gln-lys(Mtt)-ser(tBu)-gln-arg(Pmc)-ser(tBu)-gln-asp(tBu)-glu(resin)-asn-pro-val-NH\(_2\), where the groups in parentheses denote the side chain protecting groups. Next, hexafluoroisopropanol was used to cleave the peptide from the resin and deprotect the lysine, while leaving the N-terminus, the arginine, the aspartic acid, and the serines protected. Then, a selective cyclization reaction was performed to join the lysine with the glutamate without the interference of the N-terminus or aspartic acid. Finally, the cyclic peptide was fully deprotected using TFA.
The previous example used a resin which upon cleavage provides an acid functional group. However, if a resin is used that upon cleavage provides an amide functional group, then a resin can be used as a side chain protecting group for asparagines or glutamines. Scheme 2-4 highlights this subtlety. The synthesis on the left is analogous to the cyclic RGD peptide synthesis performed by McCusker et al. [2.38]. The only difference for the scheme on the right in Scheme 2-4 is that the Knorr resin is used instead of the Wang resin. In both cases, Fmoc-protected α-allylic ester aspartic acid is loaded onto the resin, followed by solid phase synthesis of the rest of the peptide (Scheme 2-4A and 2-4B). The C- and N-termini are deprotected using Pd(PPh₃)₄ and piperidine, respectively (Scheme 2-4C). Then, the cyclization is performed using any of the reagents mentioned in Section 2.4 (Scheme 2-4D). Finally, the peptides are cleaved using TFA (Scheme 2-4E). The peptide on the right now has an asparagine, because the Knorr resin was used instead of the Wang resin. Li et al. also created a C-terminal asparagine by loading a Boc-protected α-benzyl ester aspartic acid onto an MBHA resin [2.39]. This strategy was also applied to Fmoc-chemistries using the PAL resin [2.40, 41].

2.8. Non-natural Amino Acids

Glycine, alanine, valine, leucine, isoleucine, proline, and phenylalanine do not have any functional groups other than aliphatic or aromatic ones and, therefore, are not protected. But, what if a desired peptide contains only these amino acids and no amino
Scheme 2-4. Two examples of using a resin as a side chain protecting group.
acids (like lysine) with useful functional groups for bioconjugation, and the peptide is cyclic so the termini are not available? A large selection of protected amino acid derivatives based on the naturally occurring twenty amino acids, such as hydroxyproline, p-chlorophenylalanine, or proparglycine are commercially available. One of these amino acid derivatives could replace an amino acid in the desired sequence; thus, inserting a reactive functional group into the peptide. However, special care must be taken when replacing an amino acid in a biologically active sequence so that biological activity is not affected. Fmoc-protected non-natural amino acids that are not based on any of the naturally occurring twenty like diaminobenzoic acid are even commercially available.

2.9. Examples of ECM functionality in Biomaterials in Non-well-defined Manner

Pompe et al. used a common approach for the biofunctionalization of a material surface [2.42]. They copolymerized maleic anhydride with other common polymers, and used the anhydride functionality to bioconjugate fibronectin (FN) to the material surface. FN is coupled to the surface through the N-terminal amine or any lysine ε-amine. A strength of this technique is that the whole protein is used, which can help to keep the active peptide sequences within FN in the proper conformation, thus enhancing activity. However, since any lysine can react with maleic anhydride, orientation cannot be controlled, and biologically active sequences of FN may not be accessible for receptor binding, thus possibly decreasing activity. For example, lys1717 within the 13th type III domain of FN (FN-III13) is necessary for complete interaction with endothelial cell (EC)
heparin sulfate proteoglycans [2.43], and if this amino acid were conjugated to the material surface EC cell adhesion would be affected. In other examples for nonspecifically coupling proteins to materials, Cutler and Garcia also coupled a recombinant fragment of FN to an adsorbed surface of albumin using a homobifunctional linker to link amines together or a heterobifunctional linker to link amines to sulfhydryls [2.44], and Yu and Bellamkonda use carbonyldiimadazole to couple agarose gels to amino acid side chains in laminin [2.45].

Small peptides have also been haphazardly coupled to material surfaces. In one example, Mann and West modified the amino termini of RGDS and KQAGDV with succinic anhydride, introducing an acid functionality [2.46]. Using carbodiimides, these peptides were coupled to a surface containing amine containing self assembled monolayers (SAM). These peptides have more than one carboxylic acid: the succinate, the aspartate, and possibly a carboxylate C-terminus, all of which could react with the SAM, resulting in a random orientation of the peptide on the surface and possible blocking of the RGD and AGD active sequences. The AGD peptide also contains a lysine, whose ε-amine could also react with any carboxylates.

Another approach for introducing extracellular matrix (ECM) functionalities onto biomaterials is adsorption. Liu et al., Humphries et al., and Huhtala et al. adsorbed fragments of FN onto tissue culture polystyrene and observed favorable cell growth and spreading [2.47-49]. This technique requires that the material is hydrophobic enough to ensure strong/permanent adsorption otherwise the protein fragment may eventually desorb from the surface depriving cell of their necessary surface anchorage. Proteins and their fragments may also change their conformation or unfold upon surface adsorption,
which would negatively affect their biological activity. This directly correlates with results from Garcia et al. where it was found that affinity for antibodies to FN and cell differentiation of cells grown on FN varied depending on to which surface FN was adsorbed [2.50]. In addition, when using protein adsorption as a surface modification, orientation of the protein cannot be controlled so that the active sequences within the protein may face toward the material surface and not into solution for binding with cell receptors.

Electrospinning uses a macromolecule solution to create a random meshwork of ~100nm fibers. Recently, biomacromolecules such as collagen [2.51-56], elastin [2.53, 54], and fibrinogen [2.57] have been subjected to electrospinning. Subjecting fibronectin to electrospinning should be possible using analogous methods. The advantage of these materials is that they consist of an entangled fibril meshwork that can be handled and sewn to create biomaterial patches or even vascular grafts. The mesh consists of whole biological proteins, meaning that any cell adhesive peptide sequences are also included in the material. However, fluorinated alcohols used in the procedure may act as a chaotropc solvent and denature the protein possibly deactivating cell adhesive peptide sequences. The process of creating fibrils by electrospinning is much different from in vivo fibronectin fibrillogenesis [2.58], so adhesive sequences in an electrospun fibril may be obscured or may not be in the proper orientation.
2.10. Examples of Common Peptides in Biomaterials in a Well-defined Manner

GRGDSPA is a very common peptide motif in biomaterials [2.59, 60]. RGD peptides have only one amine which is located at the peptide’s N-terminus. This lone amine is frequently exploited when selectively bioconjugating RGD peptides to biomaterials. Irvine et al. modified a copolymer of methyl methacrylate and hydroxy-poly-(oxyethylene) methacrylate with RGD peptides [2.61]. Succinic anhydride was coupled to the hydroxyl groups to introduce a carboxylate functionality, which was then activated using a carbodiimide to form a succinimidyl ester. The RGDSP peptide was then added, coupling its N-terminus to the polymer. The aspartate and C-terminus did not participate in the coupling reaction, because they were not preactivated like the carboxylate in the methacrylate copolymer.

The RGD peptide has been incorporated into alginate hydrogels [2.62]. The amine terminus of the RGD peptide was coupled to carboxylates within the alginate carbohydrate using a carbodiimide and N-hydroxysuccinimide. The aspartate does not participate in the reaction since carbodiimide activation of the alginate is performed before the peptide is added. This reaction does have the potential for side reactions where the activated carboxylate can react with hydroxyls or other carboxylates in alginate to form esters and anhydrides, respectively. Since the reaction is in aqueous media, the esters will eventually succumb to hydrolysis, and the anhydrides can either react with the peptide N-terminus or immediately succumb to hydrolysis. But, the side reactions would prevent 100% conversion of coupling carboxylates to peptides.
By incorporating cysteine into an RGD peptide selective conjugation to a material surface can be achieved using gold-thiol chemistry. Ferris et al. coated titanium with gold using an electron beam evaporator, and the sulfhydryl in the RGDC peptides bonds with the gold [2.63].

Samuel Stupp’s group at Northwestern University has a unique approach to creating a material containing cell adhesive peptides. The peptide is synthesized using standard solid phase synthetic methods, and coupled to the N-terminus of the peptide is an alkyl segment; thus creating a peptide amphiphile (PA) [2.64]. These PAs self assemble into a supramolecular structure of nanofibers. If cysteines are added to the sequence, the supramolecular assembly can be crosslinked [2.64]. The PA assembly can also be performed in a cell suspension, thus trapping cells within the 3-dimensional matrix [2.65]. The RGDS peptide sequence from FN has been incorporated into theses materials [2.64], as well as the IKVAV sequence from laminin [2.65]. Using the pseudo-orthogonal Mtt protected lysine, branched functionalities such as RGD-PHSRN [2.66, 67] or IKVAV-YIGSR [2.67] have been introduced. A PA with a cyclic peptide has also been synthesized through the utilization of the orthogonal allyl ester protecting group [2.67]. Unfortunately, cell data on these materials is currently limited.

The research groups of Tirrell and Fields also synthesized peptide amphiphiles by reacting a lipid to the N-terminus of resin-supported side-chain protected peptide [2.68]. PAs were used to modify surfaces with LB (Langmuir-Blodgett) films.

Enzymatic activity has also been used to incorporate adhesive peptides into a fibrin material [2.69]. RGD peptides and cyclic RGD peptides containing the coagulation factor XIIIa recognition sequence, LNQEQVSP, were synthesized by standard solid
phase peptide synthetic methods. The peptide was incorporated into a fibrin clot in the presence of factor XIIIa and fibrinogen.

Our laboratory has also exploited the lone amino group in RGD peptides [2.70]. Here, excess glutaric dialdehyde is reacted with the peptide’s amino terminus. Following HPLC purification of the aldehydic peptide from glutaric aldehyde, the peptide is coupled to amines in poly(vinyl amine). I also designed a synthetic scheme for Shuwu Wang for conjugating poly(vinyl amine) (PVAm) to an RGD peptide separated by a poly(ethylene oxide) (PEO) diacid spacer [2.71]. The design criterion was to conjugate the PEO carboxylate to the PVAm, while not allowing aspartate’s carboxylate to participate in the reaction. This is depicted in Scheme 2-5. First, the peptide is synthesized on a Knorr resin (Scheme 2-5A). Next, after the final Fmoc deprotection, PEO diacid (in excess) is coupled to the N-terminus using EDC and NHS (Scheme 2-5B). The PEO-peptide conjugate is deprotected and cleaved from the resin using 85% TFA (Scheme 2-5C). Notice that Aspartic acid’s protecting group, the benzyl ester (-OBzl), still remains. Then, the carboxylic acid at the end of the PEO chain in the PEO-peptide conjugate is coupled to amines in PVAm (Scheme 2-5D). Alkyl acids are also coupled to PVAm. Finally, the –OBzl group is removed using either HF, TMFSA, or TMSOTf (Scheme 2-5E).

Maleimide can be specifically coupled to cysteines. Rezania et al. inserted a terminal cysteine into RGD and FHRIKKA, a heparin-binding peptide [2.72]. They modified quartz and titanium dioxide surfaces with an amine-containing SAM. The amine SAM was then coupled to the succinimidyl activated carboxylate of a heterobifunctional linker, which also contained a maleimide group. The maleimide was
Scheme 2-5. Synthesis scheme for incorporation of an RGD peptide into a surfactant polymer using a PEO diacid spacer. The “■” shows protecting groups that are removed by 85% TFA. The “●” depicts an –OBzl pseudo-orthogonal protecting group.
then selectively coupled to the lone cysteine in the peptide, thus conjugating the peptide to the surface. The maleimide-cysteine coupling is useful when the peptide contains more than one amine, especially from multiple lysines, that are critical for bioactivity. This strategy has been used with FHRIKKA, a heparin-binding peptide and IKVAV, a laminin-derived peptide [2.73, 74].

Other cell adhesive peptides from FN (REDV and LDV) and laminin (YIGSR) can be easily incorporated through their amino termini, since these peptides, like RGD, do not contain a lysine. Li et al. reacted the YIGSR amine terminus with acryloxysuccinimide, introducing an acrylate functionality, which was subjected to free radical copolymerization with N-isopropylacrylamide and acrylic acid [2.75].

A synthetic scheme was designed to incorporate heparin binding peptide 3 (HBP3, GSSGSPPRRARVTDATETTITISWRTKT) into a surfactant polymer for biomaterial surface modification (Scheme 7-2). The synthesis is discussed in Section 7.3. This particular synthetic scheme highlights how one particular carboxylic acid in poly(ethylene oxide) at the N-terminus of the peptide can be conjugated to amine functionalities in poly(vinyl amine) while the lysine ε-amino, the aspartate β-carboxylate, and the glutamate γ-carboxylate are protected and do not participate in the reaction.

2.11. References


2.71. Wang, S., Biomimetic Fluorocarbon Surfactant Polymers Designed for use on Small Diameter Eptfe Vascular Graft in Department of Biomedical Engineering, 2004, Case Western Reserve University: Cleveland, OH. p. 203.


3.1. Summary

We report on a novel series of biomimetic polymers exhibiting interfacial properties similar to the extracellular matrix. A series of well-defined surfactant polymers were synthesized by simultaneously incorporating arginine-glycine-aspartic acid (RGD) peptide, dextran oligosaccharide, and hexyl ligands with controlled feed ratios onto a poly(vinyl amine) (PVAm) backbone. The peptide sequence was H-GSSSGRGDP-SPA-NH₂ (Pep) having a hydrophilic extender at the amino terminus and capped carboxy terminus. The peptide-to-dextran ratios were varied to create surfactants having 0, 25, 50, 75, and 100 mol-% peptide relative to dextran. The surfactants were characterized by IR, NMR and AFM for composition and surface active properties. AFM confirmed full surface coverage of PVAm(Pep) (100%) on graphite, and supported the mechanism of interdigitation of hexyl ligands between surfactant molecules within a specified range of hexyl chain densities. The attachment and growth of human pulmonary artery endothelial cells on the PVAm(Pep)(100%) surface was identical to the fibronectin positive control. Cell adhesion decreased dramatically with decreasing peptide density on the surfactant polymers.
3.2. Introduction

Biomaterials are limited in long-term cardiovascular device applications because of unfavorable surface interactions with blood components [3.1]. Non-specific protein adsorption initiates the blood coagulation cascade and produces attachment sites for platelets to adhere and activate. Uncontrolled procoagulant activity at the biomaterial interface with blood leads to surface-induced thrombosis and thromboembolism, with the eventual need for surgical intervention [3.2].

Endothelial cell seeding represents an ideal method for surface coating of devices such as vascular grafts and possible prevention of thrombosis [3.3]. However, minimal vascular endothelial cell attachment and retention is observed in vivo after seeding currently used polymer grafts, due to the dependence of specific adhesive proteins for cell attachment [3.4]. Even with the pre-treatment of vascular grafts with bioactive coatings such as basement membrane gel or the cell adhesive protein fibronectin, in vivo cell attachment is only moderate [3.5], presumably because the adsorbed cell adhesion protein is denatured and/or weakly adhered cells have sloughed off due to shear forces. In vitro studies have confirmed in vivo observations reporting loss of cells from fibronectin-coated poly(tetrafluoroethylene) [3.6] and Dacron [3.7] samples after applied shear stress. This suggests the importance of incorporating stably bound ligands that remain intact and biologically active under physiologic shear forces.

A recent approach to promote endothelialization of biomaterials is the engineering of stable surface coatings that mimic the cell adhesive properties of the extracellular matrix (ECM). The ECM comprises a collection of cell adhesion proteins, proteoglycans
and glycosaminoglycans, whose composition and spatial arrangement directly affect cell adhesion, growth and proliferation [3.8]. The most ubiquitous cell adhesion sequence is the arginine-glycine-aspartic acid (RGD) motif, found in many ECM proteins such as fibronectin, vitronectin and laminin [3.8]. The RGD peptide has been immobilized directly onto many materials [3.9] including polyurethanes [3.10], poly(γ-methyl L-glutamate) [3.11], poly[(lactic acid)-co-lysine)] [3.12], and self-assembled monolayers [3.13]. Others have incorporated the peptide into alginate or poly(ethylene oxide) derived hydrogels [3.14], collagen/glycosaminiglycan matrices [3.15], or cross-linked hyaluronic acid [3.16]. In all studies the growth of endothelial cells was observed, confirming the requirement of cell adhesion peptides to promote cell growth.

As an extension of our earlier reports [3.17], we have created a model of a new class of biomimetic materials that function to create an extracellular matrix-like surface suitable for endothelial cell growth and proliferation (Figure 3-1). The surfactant polymers are composed of a poly(vinyl amine) (PVAm) backbone and hydrophilic (RGD peptide, dextran oligosaccharide) and hydrophobic alkyl ligands. The surfactant polymers are designed to prevent non-specific protein adsorption through the hydrated oligosaccharides, and to promote stable cell adhesion through the incorporated RGD peptides. Additionally, the surfactant architecture can be controlled by the molar feed ratios of hydrophilic and hydrophobic groups to maximize surface stability and cell attachment.
Figure 3-1. Molecular model of a peptide surfactant polymer (only a short segment is shown) consisting of poly(vinyl amine) (PVAm) backbone with peptide (Pep), dextran oligosaccharide (Dex) and hexyl (Hex) branches coupled to the polymer chain, designated PVAm(Pep:Dex:Hex)(2:2:6) or PVAm(Pep)(50%). The attachment of Hex, Pep, and Dex to the PVAm backbone of the surfactant polymer is random (see Table 3-1 for branch ratios in the polymer composition).
3.3. Experimental Part

3.3.1. Materials

N,N-dimethyl formamide (DMF), dichloromethane (DCM), 20% piperidine/DMF, diisopropylethanolamine, trifluoroacetic acid (TFA), O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and resin (FMOC-PAL-PEG-PS; resin cleavage site produces amide cap group at carboxy terminus) were purchased from Perseptive Biosystems. The 9-fluorenylethoxycarbonyl (Fmoc)-protected amino acids were obtained from AnaSpec. The cleavage reagents (phenol, 1,2-ethanedithiol (EDT), triisopropylsilane (TIS)), sodium cyanoborohydride (NaCNBH₃), glutaraldehyde and hexanal were used as received from Aldrich. The oligosaccharide dextran (Mₙ~1,600; polydispersity index = 1.16) was obtained from Fluka. Ultrapure water was obtained from a Millipore-RO system, with hydrocarbon content < 5ppb. Poly(vinyl amine) (PVAm) (Mₙ~6,000) was prepared and characterized as described elsewhere [3.17]. Briefly, PVAm was synthesized by radical polymerization of N-vinylformamide, hydrolysis in strong NaOH solution, and exchange with strong anionic resin. All other reagents were used as received unless otherwise stipulated.

3.3.2. Methods

Preparation of the surfactant polymer products like the model shown in Figure 3-1 involved three synthesis steps: peptide synthesis, creating an aldehyde functional group
on the peptide, and synthesis of surfactant polymer by simultaneous coupling of hydrophilic (peptide, dextran oligosaccharide) and hydrophobic (hexanal) species to PVAm.

3.3.2.1. Peptide Synthesis

The cell adhesive peptide, H-glycine-serine-serine-glycine-arginine-
glycine-aspartic acid-serine-proline-alanine-NH₂ or H-GSSSGRGDSPA-NH₂ (Pep), was synthesized using a Millipore (model 9050 plus) solid phase peptide synthesizer utilizing 9-fluorenylmethoxycarbonyl (Fmoc) methodology and standard procedures throughout the synthesis [3.18]. After synthesis, the peptide-resin was washed with DCM, exchanged with HPLC-grade methanol, transferred to a 50 mL polypropylene Falcon tube, dried under vacuum and stored at 4°C. The peptide was cleaved from the resin using a modified Reagent K cocktail [3.19], which included 75 mg phenol for 1 mL solution of TFA:water:EDT:TIS (82.5:5:2.5:1). The cocktail was added as 1.5 mL solution/100 mg peptide-resin and reacted for 2 h. Subsequent filtration into ether and three centrifugation (2100 rpm, 3 min)-ether washes yielded a white, odorless, amide-capped peptide. Reverse phase-HPLC with a preparatory scale column using a 26% - 41% acetonitrile (0.082% TFA) gradient in water (0.1% TFA) for 45 min yielded a final product of high purity (>95%).
3.3.2.2. Peptide-Aldehyde

The standard Schiff base reaction was used to create the aldehyde functional group on the peptide [3.20]. Glutaraldehyde (0.256 mmol) and NaCNBH3 (0.0512 mmol) were dissolved in ultrapure water (3 mL). A peptide solution (0.0512 mmol) in 1 mL ultrapure water was added dropwise. The solution pH was adjusted to 6.0, and the reaction was stirred at room temperature for 4 h. The N-terminal aldehyde-peptide (HCO-(CH2)4-Pep-NH2) was purified from excess glutaraldehyde by reverse phase-HPLC, using equivalent conditions to those for purification of the original peptide.

3.3.2.3. Surfactant Polymer

A series of PVAm(Pep:Dex:Hex) branched surfactant polymers were synthesized, as shown in Scheme 3-1. The molar feed ratio of Pep:Dex in the surfactant syntheses was varied between 100% Pep, which has 0% Dex, and 100% Dex, which has 0% Pep, to create a series of surfactants with different, but controlled, ratios of peptide and dextran groups on the polymer backbone. For clarity, surfactant polymers are designated by their molar amount of peptide groups, i.e., PVAm(Pep)(25%) refers to a surfactant with 25% Pep and 75% Dex in the reaction mixture. The hexyl (Hex) component is not included in the name, but every surfactant synthesis incorporated hexanal in a molar feed ratio of 2 hydrophilic species to 3 hexanals. To synthesize PVAm(Pep)(50%), peptide (HCO-(CH2)4-Pep-NH2) (0.01 mmol), dextran (Dex) (0.01 mmol) and NaCNBH3 (0.05 mmol) were dissolved in water (1 mL). Hexanal (0.03 mmol) in 0.1 mL ethanol was added to
Scheme 3-1. Synthesis of a PVAm(Pep:Dex:Hex) surfactant polymer.
this solution. Finally, PVAm (0.05 mmol) in 1 mL ethanol was added to the reaction flask dropwise and the solution stirred for 24 h at room temperature. The final product was recovered after extensive dialysis, using Spectra Por 3 regenerated cellulose membranes with 5000 molecular weight cutoff and lyophilization. This same procedure was followed to create PVAm(Pep)(100%), PVAm(Pep)(75%), PVAm(Pep)(25%) and PVAm(Pep)(0%).

3.3.2.4. Characterization Methods

FTIR transmission spectra were collected using a BIO-RAD 575 spectrometer utilizing a DTGS detector. The samples were pressed into KBr pellets before 100 scans were co-added from 4000-400 cm\(^{-1}\) at 8 cm\(^{-1}\) resolution. The spectra were normalized to the hexyl and PVAm methylene stretch at 2910 cm\(^{-1}\), which were taken to be constant for all peptide surfactants. \(^1\)H-NMR spectra were recorded using either a 200 MHz Varian XL-200 (not shown) or a 600 MHz Varian Unity INOVA spectrometer. Samples were dissolved in DMSO-\(d_6\) or D\(_2\)O to make concentrations of 7-10 mg/mL, and 64 scans were collected at room temperature. Molecular weight analysis was performed using a PerSeptive Biosystems matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-MS) using a Voyager Biospectrometry Workstation. MALDI-MS has a mass accuracy of 0.01-0.1%. Samples (0.1-0.3 mg) were dissolved in water:acetonitrile (1:1) and added to equal volume of 10 mg/mL \(\alpha\)-cyano-4-hydroxycinnamic acid in water:acetonitrile (2:3) and 0.3% TFA.
The RGD peptide activity was calculated by using an assay for the inhibition of 125I-fibrinogen binding to adenosine diphosphate (ADP)-activated platelets by the peptide [3.21]. ADP-activated platelets were incubated with 125I-fibrinogen in the presence or absence of various concentrations of the active peptide or a scrambled control peptide (GRGESP, Anaspec, Inc., San Jose, CA) in Tyrode buffer for 30 minutes. The peptides in solution then compete with 125I-fibrinogen for binding to αIIbβ3 on the platelets. (Radioactivity was then measured for the pelleted, washed platelets.) The peptide concentration required for 50% inhibition of 125I-fibrinogen binding to activated platelets (IC50) was calculated from the binding curve.

Octadecyltrichlorosilane (OTS)-modified glass coverslips were used as a hydrophobic substrate for surfactant polymer adsorption. Circular disks (15 mm) were sonicated in chloroform for 20 min, dried and placed in three small glass petri dish covers. Samples were exposed to radio frequency-induced argon plasma (Branson, 0.5 torr, 50 W forward power, 0 W reflected power) for 15 min on each side to further clean and hydroxylate the surface. Cleaned disks were placed in a large glass petri dish having a solution of dicyclohexyl (10 mL) and OTS (0.1 mL), flipped after 15 minutes and sonicated three times in fresh chloroform for 15 min. The typical water contact angle for the OTS-glass ranged from 105 - 110°.

Surfactant stability on hydrophobic substrates was tested in a flow system, followed by contact angle analysis using a Rame-Hart contact angle goniometer. Each surfactant was first dissolved in a water/acetonitrile mixture, then the OTS-glass was submerged in the solution for 24 h adsorption. The sample was removed, and dried overnight before advancing and receding water contact angle measurements were taken.
The surfactant-coated OTS was then placed in a low flow chamber for 24 h, creating conditions for maximal surfactant polymer desorption from the OTS surface. Advancing water contact angle measurements were collected by using the sessile drop method by placing five successive 2 µL drops on the surface and recording the contact angle after each drop. Receding angle measurements were taken by reading the contact angle after withdrawal of 2 µL volumes from the remaining drop.

Surface-induced assembly of surfactant polymers was analyzed on a nanoscale using atomic force microscopy (AFM). For this purpose, a Nanoscope III (Digital Instruments) AFM was equipped with a fluid cell accessory and silicon nitride cantilevers having a nominal spring constant of 0.58 N/m. Images were collected on highly oriented pyrolytic graphite (HOPG), an atomically smooth, hydrophobic surface. The aqueous surfactant solutions (0.1 - 0.5 mg/mL) were added without tip withdrawal, and images were collected at intervals to observe time dependent surface assembly. Images were obtained using tapping mode with scan rates of 1-5 Hz, typically applying tip forces of 3-5 nN.

Bioactivity of surfactant polymers was determined from in vitro cell adhesion and growth experiments using human pulmonary artery endothelial cells (HPAEC). HPAEC were harvested, subcultured and grown to confluence in 1 µg/cm² fibronectin-coated flasks in MCDB 131 (Sigma, St. Louis, MO) with 0.015% endothelial cell growth factor supplement (ECGS, Core Facilities Laboratory, Cell Biology Dept, Cleveland Clinic Foundation), 0.009% heparin (isolated from porcine mucosa, Sigma) and 10% fetal bovine serum (Sigma). Glass was coated with fibronectin (FN) and OTS-glass with surfactant polymers (1 mg/mL, 24 h) before ethylene oxide gas sterilization. HPAEC
were seeded at a density of 5000 cells/cm² and incubated at 37°C in humidified air with 5% CO₂. Prior to fixing, phase contrast images were taken of the cells on the different surfaces. For the focal adhesion studies, EC-seeded coverslips were prepared essentially as reported earlier [3.22]. Cells were fixed at 3 and 24 h and blocked with 4% horse serum in tris-buffered saline (TBS) at room temperature for 3 h. Fixed cells were incubated for 1 h in 1:1000 dilution of primary monoclonal antibody against vinculin (hVIN-1, Sigma) in TBS with 4% horse serum, washed with TBS and stained with a mixture of rhodamine phalloidin (Molecular Probes, Eugene, OR) to tag actin stress fibers and biotinylated anti-mouse IgG secondary antibody and streptavidin-FITC to tag focal adhesion molecule vinculin. Cells were analyzed with a Bio-Rad MRC 600 scanning confocal microscope, collecting four fields for each sample. In the optical images, actin stress fibers are shown as red and focal adhesion molecule vinculin as green.

Molecular modeling analysis was performed with the aid of Insight II (MSI, Inc.) molecular modeling software running on an Octane2 workstation (Silicon Graphics Inc.). Using this software, an effort was made to understand the molecular dimensions of the PVAm assembly and then to create a model to estimate the densities of peptide in the surfactant polymers’ surface coatings.
3.4. Results

3.4.1. Polymer Characterization

The composition of the purified peptide was confirmed using $^1$H NMR and mass spectrometry. All amino acid protons were identified from the 600 MHz NMR spectrum (Figure 3-2) with the exception of amide protons, whose rapid exchange with deuterium oxide prevented detection. Composition was further confirmed by MALDI-MS (Figure 3-3), with only one major peak at $m/z = 977.8$ (M+H), which is within the experimental error, being one atomic unit more than the expected value of $m/z = 976$ (M+H). All other peaks were negligible in comparison, which confirmed successful purification — a reasonable assumption because analogous peptides (those containing amino acid deletions or still containing side-chain protecting groups) have similar chemical compositions and will go into the gas phase similarly to the GSSSSGRGDSPA peptide. When these analogous peptides are present they have appeared in the spectrum.

The solution activity of the peptide was quantified from an inhibition assay of $^{125}$I-fibrinogen binding to ADP-activated platelets, as shown in Figure 3-4. The solution activity of the novel RGD-peptide ($IC_{50} = 250 \times 10^{-6}$ M) was similar to that of a positive control peptide, GRGDSP ($IC_{50}=125 \times 10^{-6}$ M). Both of these peptides exhibited higher activities than a purified fibrinogen gamma-chain fragment. The negative control peptide, RGESP, showed no inhibition activity with increasing peptide concentration, demonstrating the importance of the active RGD motif.
Figure 3-2. $^1$H NMR (600 MHz) spectra of RGD peptide (GSSSGRGDSPA) in D$_2$O with all proton peaks identified.
Figure 3-3. MALDI-mass spectrum showing single m/z peak at 977.8, corresponding to pure peptide.
Figure 3-4. Plot of inhibition of $^{125}$I-fibrinogen binding to activated platelets using various RGD controls and the novel peptide. The novel RGD peptide (IC$_{50} = 250 \times 10^{-6}$ M) was shown to have similar activity to the positive control RGDSP (IC$_{50} = 125 \times 10^{-6}$ M).
The active peptide was incorporated into peptide surfactant polymers with varying percentages of peptide and dextran oligosacharide on the polymer chain and characterized for final structure. A quantitative measure of the final molar ratio of hydrophilic and hydrophobic ligands was made using $^1$H NMR, as shown for PVAm(Pep)(100%) in Figure 3-5. The major peaks for the PVAm backbone were identified in earlier work [3.17], which provided an accurate marker for the designation of other proton shifts. The methylene and methyl groups in the hexyl ligand were also identified, and the remaining peaks were assigned to the peptide. Also important, the proton peak corresponding to the unreacted amine groups of the polymer chain was identifiable at ca. 2.6 ppm, adjacent to the DMSO solvent peak at 2.5ppm. The integrals of the proton peaks were used to determine the ratio of ligands on the surfactant polymers. For example, PVAm(Pep)(100%), with a molar feed ratio of 2:3 for Pep:Hex, was calculated to have a final molar composition of 2:3.5 for Pep:Hex. Calculated from the NMR data, Table 3-1 gives many critical parameters, such as the measured molar composition of the surfactant polymers, and also includes an estimation of the peptide surface densities of the surfactant polymer modified surfaces via the method described by Sagnella et al. [3.23].

Further insight into the coupling procedure was gained from characterization by IR spectroscopy. For each normalized surfactant spectrum (original spectra not shown), the logarithm of the ratio of the Amide I (1650 cm$^{-1}$) and strongest hydroxyl (1017 cm$^{-1}$) bands were plotted against the molar percent peptide for each surfactant, as shown in Figure 3-6. The increasing trend indicated that control of the final surfactant polymer
Figure 3-5. $^1$H NMR (600 MHz) spectra of PVAm(Pep)(100%) in DMSO-$d_6$. The major peaks of the polymer backbone, alkyl side chains and spacer are identified in the spectrum and chemical structure. Calculations of the final surfactant ligand ratios were made by integration of the peaks.
Table 1. Compositions for PVAm(Pep:Dex:Hex) surfactant polymers (Pep for peptide, Dex for dextran, Hex for hexyl ligand).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Molar feed ratio (Pep:Dex:Hex)</th>
<th>Measured ligand ratio (Pep+Dex:Hex)</th>
<th>Theoretical max composition</th>
<th>Measured composition</th>
<th>Estimated no. of gp. per surfactant</th>
<th>Estimated peptide surface density</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mol-%</td>
<td>mol-%</td>
<td>Pep</td>
<td>Dex</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pep</td>
<td>Dex</td>
<td>Hex</td>
<td>Am</td>
</tr>
<tr>
<td>Pep(100%)</td>
<td>4:0:6</td>
<td>1:1.7</td>
<td>33</td>
<td>0</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>Pep(75%)</td>
<td>3:1:6</td>
<td>2.0:1</td>
<td>25</td>
<td>8.3</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>Pep(50%)</td>
<td>2:2:6</td>
<td>3.2:1</td>
<td>17</td>
<td>17</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>Pep(25%)</td>
<td>1:3:6</td>
<td>4.2:1</td>
<td>8.3</td>
<td>25</td>
<td>50</td>
<td>17</td>
</tr>
<tr>
<td>Pep(0%)</td>
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<td>1:3.7</td>
<td>0</td>
<td>33</td>
<td>67</td>
<td>0</td>
</tr>
</tbody>
</table>

a) Calculated from NMR spectra taken in D₂O or DMSO-d₆. Measured ligand ratios refer to peptide and dextran groups combined vs hexyl groups.
b) After Qiu et al.[17b]
c) Assumes 140 monomer units based on PVAm Mₙ = 6,000.
d) After Sagnella et al.[23]
Figure 3-6. Absorbance ratios for PVAm(Pep:Dex:Hex) surfactant series. Plot of logarithm of absorbance ratio (amide I:hydroxyl) against mole percent peptide in the peptide surfactants with 0, 25, 50, 75, and 100% peptide. All IR spectra were normalized to the methylene peak (of hexyl groups and PVAm) at 2910 cm\(^{-1}\), which is assumed to be constant. The relative absorbance for amide I (1650 cm\(^{-1}\)) and the strongest hydroxyl peak (1017 cm\(^{-1}\)) were ratioed and plotted as log ratio versus mole percent peptide. The increasing trend demonstrates synthetic control over the final surfactant structure.
composition was achieved through the input parameters of the molar feed ratios of the ligands.

3.4.2. Surface activity of surfactant polymers

The surfactant polymers were shown to be surface active on OTS-glass. For all peptide surfactants, the advancing water contact angle dropped from 110 degrees to 20-30º, which indicated adsorption of the surfactants to the surface. After 24 h desorption and drying of the sample, the advancing water contact angles for all surfactants showed only slight increase to 30-40º, indicative of surfactant stability on the hydrophobic substrate.

A more detailed understanding of the peptide surfactant adsorption and assembly onto hydrophobic surfaces was obtained by AFM. Two AFM images of PVAm(Pep)(100%), taken after 25 min and 2.5 h are presented in Figure 3-7 (left) and (right), respectively. Distinct ordered regions of surfactant adsorption from 1 mg/mL solutions were visualized after 25 min. The surfactant adsorbed on HOPG in preferential directions rather than randomly on the surface. This epitaxial ordering of peptide surfactant polymer was in agreement with similar surfactant systems imaged by AFM on HOPG [3.17]. Complete monolayer coverage by PVAm(Pep)(100%) on the HOPG surface was obtained after 2.5 h.

Endothelial cell adhesion, growth and proliferation, on the surfactant-coated surfaces were directly related to the Pep:Dex ratio in the surfactant polymers. No cell growth was observed on surfaces with PVAm(Pep)(0%) coatings using phase contrast
Figure 3-7. Atomic force microscope images of PVAm(Pep)(100%) showing surface-induced and self-assembled adsorption processes on graphite. (A) After 25 min adsorption time, distinct regions of surfactant adsorption are seen, with the adsorbed chains aligning in particular directions. This is attributed to the epitaxial adsorption of the hexyl chains in registry with the graphite hexagonal lattice. (B) After 2.5 h adsorption time, full surface coverage is seen with very few distinct regions visible. Post-imaging analysis showed that the coating is a monolayer of ~1 nm height.
images, whereas on the PVAm(Pep)(100%) coated surfaces, cell growth was similar to
that on the positive control fibronectin-coated glass (data not shown). Cell growth was
also visualized using fluorescent staining for the actin fibers and focal adhesion points to
the surface. As shown in Figure 3-8, cell adhesion and growth improved dramatically on
surfaces with the higher Pep-Dex ratios, as seen for cells on PVAm(Pep)(100%) (Figure
3-8A) and PVAm(Pep)(75%) (Figure 3-8B) with long actin stress fibers and many focal
adhesions. As the peptide-to-dextrans ratio decreased (below 50% peptide) (Figures 3-8C-
D), the cell viability decreased as also shown more quantitatively in other studies [3.23,
3.24].

3.4.3. Molecular Modeling of Peptide Surface Densities

A model based on molecular distances was created to estimate the surface peptide
densities of the surfactant polymers’ coated surfaces. The first step was to define a unit
cell for the 2-dimensional peptide lattice: An ordered 2-dimensional lattice is described
by a parallelogram whose area is equal to the length of its base multiplied by its height.
The four corners of the parallelogram are points in the lattice, it this case, individual
RGD peptides. Each RGD peptide is in four parallelograms; therefore, each
parallelogram contains four quarters of a peptide, or one peptide in total (Figure 3-9A).
The base of the unit cell becomes the distance along the PVAm backbone between two
consecutive peptides, and the height of the unit cell becomes the distance between two
neighboring backbone chains (Figure 3-9B).
Figure 3-8. Surface-dependent cell adhesion on PVAm(Pep) surfactant polymers. (A) PVAm(Pep)(100%) shows endothelial cell attachment with numerous actin stress fibers (red) and focal adhesions seen (green, along cell periphery). As the peptide density decreases from 100 (A) to 75 (B) to 50% (C), the cell viability declines, as observed by fewer stress fibers and focal adhesions, as well as more rounded cell morphology. Below 50% peptide, cells show minimal or no cell adhesion and growth on 25% (D).
Figure 3-9. Methods to estimate peptide surface densities. (A) The black dots are lattice points represent peptides. The lattice forms a parallelogram (blue). (B) The two red lines represent two PVAm backbone chains. The base of the unit cell becomes the distance along the backbone between two consecutive peptides, and the height of the unit cell becomes the distance between two backbone neighboring backbone chains. The height of the unit cell was derived from molecular modeling measurements. The height was determined for two extreme cases: (C) full interdigitation and (D) no interdigitation.
The height of the unit cell was modeled using the molecular modeling software. Two extreme regimes were modeled: One assuming complete interdigitation of the hexyl side chains of neighboring backbones, and one assuming no degree of interdigitation of the hexyl side chains of neighboring backbones (Figure 3-9C and 3-9D, respectively). The surfactant polymers could reasonably adsorb according to either regime or at some degree in between. The distance between two neighboring backbones for the interdigitation regime was measured to be 1.3 nm. The distance between two neighboring backbones for the non-interdigitation regime was measured to be 2.1 nm.

The base of the unit cell was derived from the integration of $^1$H-NMR peaks characteristic to each type of side chain on the backbone (peptide, hexyl, or primary amine). It was determined that for the 100% peptide surfactant polymer 20% of the PVAm backbone amines were conjugated to a peptide ligand. This translates to one peptide out of every five PVAm amines. The distance along the backbone between amines was measured using molecular modeling software and found to be 0.25 nm per amine; therefore, 5 amines are 1.3 nm apart. Thus, with $B = 1.3$ nm and $H = 1.3$nm or 2.1 nm, there is one peptide for every $1.7 \sim 2.6 \text{ nm}^2$. Table 3-2 shows useful forms of this figure for easy comparison with previously published values, and the highlighted values were also inserted into Table 3-1. The values for the 25%, 50%, and 75% surfactant polymers were assumed to have molar peptide compositions along the backbone at 25%, 50%, and 75% of the 100% peptide surfactant polymer, respectively. This model has been implemented both with hydrocarbon ligands [3.23] and fluorocarbon ligands [3.25].
<table>
<thead>
<tr>
<th>Regime</th>
<th>100% polymer</th>
<th>75% polymer</th>
<th>50% polymer</th>
<th>25% polymer</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20 mol % peptides</td>
<td>15 mol % peptides</td>
<td>10 mol % peptides</td>
<td>5 mol % peptides</td>
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<tr>
<td></td>
<td>1 peptide / 5 amines</td>
<td>1 peptide / 6.7 amines</td>
<td>1 peptide / 10 amines</td>
<td>1 peptide / 5 amines</td>
</tr>
<tr>
<td></td>
<td>1 peptide / 1.3 nm (=b) along backbone</td>
<td>1 peptide / 1.7 nm (=b) along backbone</td>
<td>1 peptide / 2.5 nm (=b) along backbone</td>
<td>1 peptide / 5 nm (=b) along backbone</td>
</tr>
<tr>
<td>No interdigitation:</td>
<td>1 peptide / 2.6 nm²</td>
<td>1 peptide / 3.5 nm²</td>
<td>1 peptide / 5.3 nm²</td>
<td>1 peptide / 11 nm²</td>
</tr>
<tr>
<td>(H = 2.1 nm = inter-</td>
<td>0.38 peptides/nm²</td>
<td>0.28 peptides/nm²</td>
<td>0.19 peptides/nm²</td>
<td>0.095 peptides/nm²</td>
</tr>
<tr>
<td>backbone distance)</td>
<td>0.063 nmol/cm²</td>
<td>0.046 nmol/cm²</td>
<td>0.032 nmol/cm²</td>
<td>0.016 nmol/cm²</td>
</tr>
<tr>
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<td>63 pmol/cm²</td>
<td>46 pmol/cm²</td>
<td>32 pmol/cm²</td>
<td>16 pmol/cm²</td>
</tr>
<tr>
<td>Full interdigitation:</td>
<td>1 peptide / 1.7 nm²</td>
<td>1 peptide / 2.3 nm²</td>
<td>1 peptide / 3.4 nm²</td>
<td>1 peptide / 6.8 nm²</td>
</tr>
<tr>
<td>(H = 1.3 nm = inter-</td>
<td>0.60 peptides/nm²</td>
<td>0.44 peptides/nm²</td>
<td>0.30 peptides/nm²</td>
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<tr>
<td>backbone distance)</td>
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<td>0.073 nmol/cm²</td>
<td>0.050 nmol/cm²</td>
<td>0.25 nmol/cm²</td>
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<tr>
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<td>73 pmol/cm²</td>
<td>50 pmol/cm²</td>
<td>25 pmol/cm²</td>
</tr>
</tbody>
</table>
3.5. Discussion

The peptide sequence (-GSSSGRGDSPA-) was designed to preserve the biological activity of the peptide even in the presence of the conformational constraints caused by coupling to the polymer and the surfactant adsorption onto a biomaterial substrate. The first five amino acids, -GSSSG-, act as a hydrophilic spacer to minimize hydrophobic interaction with the surface or neighboring hexyl ligands and to allow greater conformational freedom. The amino acids following the RGDS active sequence, proline-alanine-NH₂, distance the carboxy terminus from the active site and eliminates the negative charge, creating a better peptidomimetic model of the natural RGD binding site in various extracellular matrix proteins. Some reports suggest improved activity is obtained from cyclic RGD peptides as cell integrin antagonists [3.26]. However, a linear -RGD- peptide has been shown in many surface-constrained applications to retain activity, even without incorporating spacers or carboxy capping groups, i.e., tethering only the RGD or RGDXX peptides [3.10c, 3.11, 3.27]. Given the surfactant polymer architecture and thermodynamically driven coating process, our nonapeptide design allows retained activity after coupling through conformational freedom and enhances availability of the active site by extension from the surface.

The peptide surfactant polymer series provides a well-defined synthetic approach to develop biomimetic materials for understanding the surface peptide concentrations necessary for effective endothelial cell growth and proliferation. The synthetic procedure allows for control of the hydrophilic-to-hydrophobic balance and the Pep:Dex ratio through the molar feed ratios used. Any deviation of the final molar composition from
the molar feed ratios is due to the different reactivities of the dextran lactone, peptide aldehyde, and hexanal resulting largely from the effects of differing kinetics and solvations among the ligands. These effects, however, are minimized by using the same coupling chemistry for all ligand groups (Schiff base formation and reduction) and by using hydrophilic groups of similar molecular weight. The IR results (Figure 3-6) confirms this qualitatively, as seen by the increasing trend of the peptide-to-dextran absorbance ratios of each surfactant with the increase in the molar percent peptide in the surfactant. Some difficulties arise in using NMR for quantitative ratio determination of surfactant polymers other than PVAm(Pep)(100%) and PVAm(Pep)(0%), where only one hydrophilic ligand is used, due to the overlap of proton peaks derived from peptide and dextran. However, the data in Table 3-1 suggests that the final ratios on the surfactant polymers closely match the molar feed ratios. In particular, the NMR data for the PVAm(Pep)(100%) shows very similar observed ratio (1:1.75) to the feed ratio (1:1.5). This analysis, in combination with the results from the cell adhesion data (Figure 3-8), shows a clear trend of increasing cell viability with increasing percentage of incorporated peptide. This offers some added confidence to the initial analytical results that indicate the final peptide-to-dextran ratios closely match the feed ratios.

The surface active properties of the surfactant polymers are a reflection of the hydrophobic-to-hydrophilic balance. The initial surfactant series synthesized for this study, with an overall hydrophilic-to-hydrophobic molar feed ratio of 2:3, were water insoluble, necessitating between 30-50% acetonitrile in aqueous solutions for effective solvation and subsequent surface coating. Ideally, one desires water soluble materials with strong surface adsorption characteristics. From the IR and NMR data obtained,
simple iteration of the feed ratios led to the synthesis of water soluble peptide surfactants (hydrophilic-to-hydrophobic molar feed ratio of 2:1) with full surface coverage and stable monolayer adsorption, as confirmed by AFM and contact angle data.

The AFM not only provided a molecular level time-scale for surfactant adsorption, but also a mechanism for surfactant adsorption onto a hydrophobic surface, HOPG [3.17]. PVAm(Pep)(100%), surfactant polymers adsorbed as ordered monolayers (Figure 3-7A), rather than randomly on the surface. This is attributed to a nucleating event facilitated by cooperative binding of surfactant chains with initially adsorbed surfactant molecules. Specifically, monolayer formation was accomplished through a combination of surface- and self-induced assembly. The adsorption on the graphite exhibits three-fold symmetry (Figure 3-7A), corresponding to the hexagonal lattice of the underlying HOPG substrate. There is a large enthalpic driving force (6.28 kJ/mol per methylene group [3.28]) for epitaxial alignment of the hexyl chains with the graphite, causing the PVAm backbone to be constrained to elongate in preferred directions. This suggests an energetically favorable conformation for the adsorbed hexyl chains induced by HOPG. Self assembly occurs through interdigitation of hexyl side chains of a newly adsorbed chain with an adsorbed surfactant polymer causing the "propagation" or "zipping up" effect. From a geometric analysis of similar surfactant polymers [3.29], it was estimated that between 27% and 58% of the amino groups of the PVAm chain should be occupied by alkyl chains to allow effective and rapid interdigitation of adjacent chains and the most stable adsorption arrangement. For PVAm(Pep)(100%), the hexyl chain composition was 36%, falling within the range for effective and stable adsorption. Although this study was performed using a model surface, HOPG, the hexyl density
range should be applicable for surfactant assembly on many hydrophobic surfaces, as demonstrated in our related reports [3.30].

The effect of surface peptide density on cell adhesion and morphology was demonstrated by fluorescence microscopy using fluorophores to label actin stress fibers and vinculin protein involved in focal adhesion with surfaces. Cell growth on PVAm(Pep)(100%) was essentially identical to that on the fibronectin coated positive control surface. Numerous focal adhesions confirm the specific interaction of the cell’s integrins with the RGD peptide. Recent work by Murugesan et al. [3.24] has identified the cell receptor integrins and the change in integrin binding with time of cell attachment to the surface.

The dramatic effect of the density of peptide ligands on cell morphology (Figure 3-8) was to some extent unexpected. The four PVAm(Pep) surfactants with coupled peptide ligands (25% through 100%) all had surface concentrations of peptide well above a reported minimum required for a peptide-to-peptide distance of 440 nm necessary to promote cell adhesion [3.31]. The incorporation of the dextran oligosaccharide seemingly plays a significant role in our results. The peptide was designed to have the active RGD peptide extend beyond the calculated dimensions of the hydrated dextran (see Figure 3-1). It is certainly possible that the conformations available to the dextran and peptide ligands allow for burial of the peptide or loss of peptide activity due to hydrogen bonding with neighboring dextrans. No cell adhesion was observed on the PVAm(Pep)(0%) coating, and similar results have been found on PEO hydrogels and interpenetrating networks [3.32]. This supports the interpretation of suppression of non-specific protein adsorption as reported previously [3.17]. Since endothelial cells are
dependent on matrix proteins for adhesion, no cell attachment will occur in the absence of cell-binding proteins. This suggests that the hydration of the dextran layer imparts interfacial characteristics unsuitable for cell growth beyond the effects seen for the RGD-peptide conjugated polymers.

The successful attachment and growth of endothelial cells on the extracellular matrix-like coatings demonstrates the potential usefulness of the peptide surfactant polymers. The biomimetic approach to endothelialization provides an effective means to engineer a surface suitable to grow a healthy, confluent cell layer by manipulation of the surfactant polymer side groups. One could incorporate a variety of biologically active peptide and oligosaccharide structures onto the surfactant to target (or inhibit) specific cellular responses. The ability to design well-defined cell adhesive surfaces will have future significance in understanding cell behavior and improving cardiovascular device longevity.

3.6. Conclusions

A new class of well-defined biomimetic materials were synthesized with a poly(vinyl amine) (PVAm) backbone with hydrophilic (RGD peptide, dextran oligosaccharide) and hydrophobic hexyl ligands. These PVAm(Pep:Dex:Hex) surfactants were synthesized by simultaneous incorporation of peptide, dextran, and hexyl groups, and characterized by NMR and IR to calculate the final molar ratios. Contact angle measurements confirmed surface stability of the surfactants. AFM images
supported the mechanism of cooperative binding between the hexyl groups, allowing maximal surfactant adsorption at hexyl chain densities between 27% and 58%. Cell adhesion and proliferation on PVAm(Pep)(100%) was similar to that on fibronectin-coated surfaces, with rapid decline in cell viability for surfactant polymers with peptide densities of less than 50%.

3.7. Acknowledgments

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3.8. References


Chapter 4. HEPARIN BINDING PEPTIDE SURFACTANT POLYMERS

4.1. Introduction

In 2003 coronary heart disease killed or contributed to the death of 653,000 individuals [4.1]. It is and has been the leading cause of death for over 75 years. Currently, the preferred small-diameter vessel replacements are still autogenous vessels [4.2]; however, in many patients, they are of poor quality or nonexistent due to previous procedures [4.3]. There are also complications with using veins, which have a different anatomy than that of arteries, to perform an arterial physiological role under increased flow and pressure conditions [4.4]. There is clearly a need for successful, long-term, synthetic, small-diameter, vascular grafts.

Many materials have been evaluated as grafts including, but not limited to Dacron (poly(ethylene terephthalate), PET), GoreTex (expanded poly(tetrafluoroethylene), ePTFE), polyethylene (PE), nylon, polydimethylsiloxane (PDMS), and polyurethane [4.5]. Numerous attempts have been made to modify the previously mentioned materials in an effort to improve their interfacial properties with blood. The rationale is to use a material that already possesses desirable bulk properties such as strength, durability, and compliance and then modify its surface to reduce its thrombogenicity [4.6]. Perhaps the most promising surface modification is to support a layer of vascular endothelium on the interior of the graft. The endothelium, composed of endothelial cells (EC), is the natural lining of blood vessels, and therefore has an inherent resistance to thrombosis [4.7-13].
However, human EC do not adhere to the hydrophobic surfaces of polymer grafts [4.14-18]. An interface must be created that both adheres to the polymer surface and supports EC attachment, growth, and proliferation. These interfaces usually incorporate the cell adhesive peptide, arginine-glycine-aspartic acid (RGD), which is a native sequence found many extracellular matrix (ECM) proteins, including fibronectin, laminin, collagen, and vitronectin [4.19]. One problem is that RGD is only one of many cell adhesive functionalities that ECs utilize, and a stronger EC attachment may result from the use of additional cell adhesive moieties. Also if the endothelium of a graft is damaged, exposing the RGD peptide to the blood, thrombogenicity is very likely [4.20, 21].

Heparin binding peptides (HBP) are another cell adhesive functionality that could be exploited. ECM binding through EC surface proteoglycans that contain heparan sulfate is less specific and involves ECM proteins that contain heparin-binding domains. The peptide sequences in these domains consist of clusters of basic residues spaced apart by hydrophobic residues, such as RRAR and PRPRP [4.22-27]. It is proposed that these heparin-binding interactions reinforce the integrin adhesion complexes and can supplement integrin binding [4.28]. Evidence suggests that for complete binding of EC to the ECM via the $\alpha_5\beta_1$ integrin, interactions with cell surface proteoglycans are necessary [4.29]. The heparin-binding region of FN has been attributed to the WQPPRARI, SPPRARVT, and PRRARVTDATETTITISWRTKT peptide sequences [4.30, 31].

A design of a vascular graft surface modification that will promote endothelialization is proposed. An amphiphilic polymer, which will modify a graft through a simple dip-coating process, will be synthesized (Figure 4-1). This polymer
**Figure 4-1.** Design of biomimetic surfactant polymer. Attached to a PVAm backbone (A) are hydrophobic legs (B) for adsorption onto the material surface, integrin-binding peptides (C) and HSPG-binding peptides (D) for EC attachment, and
design consists of a poly(vinyl amine) (PVAm) backbone with hexanoyl hydrophobic branches for adsorption onto the material surface. The hydrophilic groups are HBPs for promotion of EC attachment through the EC heparin sulfate proteoglycans (HSPG). The surfactant polymers are synthesized and characterized at each step by mass spectrometry, infrared spectroscopy, nuclear magnetic resonance spectroscopy, and high-pressure liquid chromatography. Secondly, the hydrophobic surfaces are prepared and coated with the surfactant polymer followed by surface characterization using contact angle goniometry. Finally, endothelial cells are seeded on the modified surfaces, and their attachment and growth are analyzed.

4.2. Materials and Methods

4.2.1. Materials

For the synthesis of poly(vinyl amine), N-vinyl formamide (Aldrich Chemical Company, Inc., Milwaukee, WI) was used as received. Azobisisobutyronitrile (Aldrich) was recrystallized in methanol (HPLC grade). The inert gases argon (ultra high purity grade) and nitrogen were obtained from Praxair (Danbury, CT). A Teflon membrane (Osmonics Inc., Minnetonka, MN) with a 5.0 µm pore size was used for filtration. Hydrochloric acid (Fisher Scientific, Springfield, NJ, ACS grade, 11 N) and sodium hydroxide (Fisher, ACS grade) were used to adjust the pH of polymer solutions. The
Amberlite IRA-400 (ammonium hydroxide functional group) (Aldrich) strong anionic ion exchange resin was rinsed with ultra pure water prior to use.

For solid phase peptide synthesis (SPPS) all protected and unprotected amino acids (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr-OH, Fmoc-Trp-OH, and Fmoc-Val-OH) were purchased from Anaspec, Inc. (San Jose, CA).  The N, N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), dichloromethane (DCM), and N, N-diisopropylethylamine (DIPEA) solvents were SPPS grade and purchased from Applied Biosystems (Foster City, CA).  The 9-fluorenylmethoxycarbonyl-PAL-polystyrene solid support resin (Fmoc-PAL-PS), the activating agent cocktail, HBTU ((2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate) with HOBt/DMF (0.5 M 1-hydroxybenzotriazole in N, N-dimethylformamide), and the cleavage/deprotection reagent, trifluoroacetic acid (TFA), were also purchased from Applied Biosystems.  The scavengers for the peptide cleavage reaction (phenol, 1,2-ethanedithiol (EDT), and triisopropylsilane (TIS)) along with piperidine were purchased from Aldrich.  All SPPS and peptide cleavage solvents and reagents were used as received.  For peptide N-terminal modification and polymer surfactant synthesis, the poly(ethylene glycol) bis(carboxymethyl) ether (3,6,9-trioxadecanedioc acid, $M_n = 222$ g /mol), hexanoic acid (Hex), and NHS (N-hydroxysuccinimide) were used as obtained from Aldrich.  The EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) was purchased from Sigma Chemical Co. (St. Louis, MO). Spectra Pro 3 regenerated cellulose dialysis membranes with 500 and 3000 molecular weight cutoffs (MWCO) were obtained from Fisher.
For FT-IR spectroscopy, potassium bromide (KBr) was IR grade and acquired from Fisher. In the nuclear magnetic resonance spectroscopy experiments deutrated water (D$_2$O) and dimethyl-d$_6$ sulfoxide (DMSO-d$_6$) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Also, α-cyano-4-hydroxycinnamic acid was obtained from Aldrich as the MALDI-MS matrix.

For the preparation of specialized surfaces, octadecyltrichlorosilane and dicyclohexyl were purchased from Aldrich and were distilled prior to use. Also, the microscope cover glass was type #1 15 mm discs, obtained from Fisher, and the Millex-GP (Millipore, Bedford, MA) sterile filters had a 0.22 µm pore size.

For endothelial cell culture, phosphate buffered saline (PBS), tris buffered saline (TBS), fetal bovine serum (TBS), type II collagenase, and heparin (isolated from porcine mucosa) were all purchased from Sigma. ACS grade sodium bicarbonate was obtained from Fisher. The trypsin/EDTA and the trypan blue were purchased from Invitrogen (Carlsbad, CA). The Core Facilities Laboratory, Cell Biology Dept., Cleveland Clinic Foundation provided the endothelial cell growth factor supplement (ECGS). Sterile cryovials, bottle top filters, and the MCDB-131 were acquired from Baxter (Deerfield, IL). For fluorescent labeling in confocal microscopy, the hVIN-1 primary monoclonal antibody was purchased from Sigma, biotinylated anti-mouse IgG secondary antibody was purchased from Amersham Pharmacia (Piscataway, NJ), and the rhodamine phalloidin and streptavidin FITC were acquired from Molecular Probes (Eugene, OR).

Ultra pure water was produced by Millipore’s Milli-RO Reverse Osmosis System and Milli-Q UV Plus Water System. In addition, all solvents, unless otherwise indicated, were purchased from Fisher and used as received.
4.2.2. Synthesis

The synthesis of a poly(vinyl amine) (PVAm) surfactant polymer is a multi-step process. A similar surfactant polymer was first made by Qiu et al. [4.32]. However, dextran sugars were used as hydrophilic ligands in creating these polymers instead of peptides. The creation of a similar surfactant polymer with peptide ligands was described in the previous chapter [4.33]. In the surfactant polymer described here, the functional peptide has a different target—the heparin sulfate proteoglycan, and the peptide is attached to PVAm via the more hydrophilic PEO spacer. The creation of this surfactant polymer required a slightly different synthetic strategy, which is shown in Scheme 4-1. First, the PVAm backbone of the surfactant polymer must be made (A). Then, the heparin-binding peptide is synthesized by SPPS (B). Upon modification of the amino terminus (C & D), the peptide along with the hydrophobic hexanoic acid ligands is grafted onto the PVAm backbone, creating a surfactant polymer (E).

4.2.2.1. Poly(vinyl amine) Synthesis

The synthesis of PVAm (Scheme 4-1 (A)) is conducted in three main steps, starting with the polymerization of N-vinyl formamide (NVF). NVF (11.4 mmol) and isopropanol (30 mL, electronics grade) were purged with argon while undergoing with liquid nitrogen three freeze/thaw cycles to eliminate oxygen. The polymerization initiator, 2, 2'-azobisisobutyronitrile (AIBN) (0.319 mmol), was added to the reaction mixture, which was then refluxed at 60°C for 4 h under argon. After 4 hours, some
Scheme 4-1. Surfactant polymer synthesis.
poly(N-vinyl formamide) (PNVF) had precipitated out of solution, and some isopropanol had evaporated. Water was added to the reaction mixture to dissolve the precipitate, and the total volume was precipitated in acetone (75 mL, ACS grade). The precipitate was then filtered using a Teflon membrane, dried overnight at 50°C at less then 5 mm Hg of pressure, and finally lyophilized. Transmission Fourier transform infrared spectroscopy (FT-IR) and proton nuclear magnetic resonance spectroscopy (¹H-NMR) were used to chemically characterize the product. The molecular weight of the polymer backbone was characterized using gel permeation chromatography (GPC).

The PNVF was subjected to basic hydrolysis, changing the formamide group to an amine salt. PNVF (2.25 mmol), ultra pure water (0.753 mL) and NaOH (3.40 mmol) were refluxed under continuous nitrogen purge at 80°C for 6 hours. Then, approximately 15 mL of hydrochloric acid was added, dropwise, to precipitate PVAm·HCl. This precipitate was filtered with a Teflon membrane, yielding a large gummy aggregate as the product. After washing with methanol (ACS grade), the product was collected and dried overnight at 50°C under 5 mm Hg of pressure, producing yellowish, sticky PVAm·HCl. FT-IR and ¹H-NMR were used to chemically characterize the product.

To convert the amine salt group of PVAm·HCl to an amine, an Amberlite IRA-400 strong ionic exchange resin was used. Hydrated resin (approximately 300 mL) was packed lightly in a column and pre-cycled by passing 200 mL of a 1 M NaOH solution, followed by flushing with ultra pure water (200 mL). Gravity flow was roughly at a rate of 1 mL/min. PVAm·HCl solution (1 mL) in water at 0.100 g/mL concentration was loaded onto the column and eluted with pure water. Only the eluate with a basic (> 7) pH (~150 mL) was collected, roto-evaporated to a workable volume (~5 mL), dialyzed for 24
h using a regenerated cellulose membrane (MWCO = 1000), and lyophilized to obtain an almost white PVAm powder, which was stored at room temperature in a desiccator. FT-IR and $^1$H-NMR were used to chemically characterize the product.

4.2.2.2. Synthesis of Peptide Ligands

The synthesis of the GSSSGWQPRAI (HBP1, Scheme 4-1 (B)) heparin-binding peptide was performed with the aid of an Applied Biosystems 433A peptide synthesizer. The amount of the Fmoc-PAL-PS resin was 0.25 mmol and the amino acids were reacted at a molar excess of 4 using the FastMoc coupling chemistry protocol with conditional conductivity monitoring of Fmoc deblocking. After the final deblocking the resin was washed with DCM and HPLC grade methanol and dried overnight at room temperature under partial vacuum.

In order to couple the N terminus of the peptide to the amine functionalities of PVAm, a functional group modification was required (Scheme 4-1 (C)). Amide coupling using EDC and NHS was used to create an N-terminal acid on the peptides. First, polyethylene glycol bis(carboxymethyl) ether (PEO, 5 mmol) was added to the resin-bound peptide (0.25 mmol), which was swelling in enough DMF to fully cover resin. Separately, EDC (1 mmol), and NHS (1 mmol) was dissolved in DMF (0.5 mL). This solution was then added dropwise to the swelled resin and allowed to react under gentle stirring for three hours at 37°C and then at room temperature overnight. The reaction solution was decanted from the resulting resin-bound PEO-HBP1, which was subjected to
further washes in DMF (2x), DCM (1x), and methanol (3x) and dried overnight at room temperature under partial vacuum.

The cleavage and deprotection of the peptide was performed with a freshly made solution consisting of TFA (86%), ultra pure water (5%), phenol (5%), EDT (3%), and TIS (1%) [4.34-36]. (Scheme 4-1 (D)) For every 100 mg of resin, 1.5 mL of the solution was required. The resin, TFA, phenol, water, EDT and TIS were allowed to react under gentle stirring. After four hours, the solution was filtered from the resin. The filtrate was precipitated in cold ethyl ether (ACS grade, 40 mL per 100 mg resin). The filtered polystyrene beads were washed with a few drops of TFA to rinse away any residual peptide. The precipitate was centrifuged (2100 rpm, 3 min.) forming a pellet, and the ether was decanted. Following three additional ether washes, the pellet was dried in a fume hood, dissolved in a small amount of water, and lyophilized. Further purification was performed by reversed-phase HPLC using a preparatory-scale C-18 column. The collected peptide was rotary evaporated to a workable volume (~10 mL), lyophilized, and then stored in a desiccator at –20°C. The resulting peptide was characterized via transmission FT-IR, 1H-NMR, two-dimensional NMR (2d-NMR), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS). The syntheses of the GSWSGSPRRARVT (HBP2) heparin-binding peptides and the RGD peptide (GSWSGRGDSPA) were preformed in an analogous fashion to yield PEO-HBP2 and PEO-RGD, respectively. Negative control HBP ligands were also created by replacing the crucial arginine residues with alanines, resulting in the following sequences:

GSSSGWQPPAAAAl (PEO-xHBP1) and GSWSGPPAAAAVT (PEO-xHBP2).
4.2.2.3. Synthesis of Peptide Surfactant Polymers

In order to synthesize the surfactant polymer, the same reaction as above, amide conjugation, is employed (Scheme 4-1 (E)). The amines of PVAm are coupled to the acid functional groups in PEO-HBP1 and in hexanoic acid. First, PEO-HBP1 (0.075 mmol), PVAm (0.19 mmol amino groups), and NHS (0.30 mmol) was dissolved in DMSO (1.0 mL). Next, EDC (0.30 mmol), dissolved in DMSO (1.0 mL) was added dropwise to the polymer/peptide solution. The reaction mixture was stirred for three hours at 37°C and then overnight at room temperature. After 24 hours, additional NHS (0.45 mmol) and EDC (0.45mmol) were added, followed by the dropwise addition of hexanoic acid (0.11mmol) in 1.0 mL of DMSO. The resulting surfactant polymer (PVAm(HBP1:Hex)[2:3], named so because its feed ratio was 2 HBPs per 3 Hex) was purified by dialysis (5000 MWCO membrane), lyophilized, and stored in a desiccator at -20°C. It was finally characterized via transmission FT-IR and $^1$H-NMR before creating modified surfaces. The same procedure was used to synthesize PVAm(HBP2:Hex)[2:3], PVAm(RGD:Hex)[2:3], PVAm(xHBP1:Hex)[2:3], and PVAm(xHBP2:Hex)[2:3]. In analogous procedures, PEO-HBP1 (0.038 mmol) plus PEO-RGD (0.038 mmol) were combined to make PVAm(HBP1:RGD:Hex)[1:1:3], and HBP2 (0.038 mmol) plus PEO-RGD (0.038 mmol) were combined to make PVAm(HBP2:RGD:Hex)[1:1:3].
4.2.3. Characterization

4.2.3.1. IR

Transmission FT-IR spectra were recorded using a BIO-RAD FTS-575C infrared spectrometer with a mid-IR DTGS detector controlled by the BIO-RAD Win-IR software. A powdered sample (0.1 mg) was ground with KBr (0.1 g), heated in an oven for five minutes to eliminate moisture, and pressed under a force of 10 tons for 10 minutes, yielding a transparent pellet. For each sample, 100 scans were co-added with a resolution of 8 cm\(^{-1}\) in the range of 4000 - 400 cm\(^{-1}\). Spectra were leveled and zeroed and normalized to make the largest peak have an absorbance of 1. The carbon dioxide absorbance at 2200 cm\(^{-1}\) was eliminated, and a water vapor spectrum was subtracted from the data.

4.2.3.2. NMR

NMR spectra were recorded using a 600 MHz Varian \textsuperscript{UNITY}NOVA spectrometer. Samples (7-10 mg) were dissolved in either D\(_2\)O or DMSO-d\(_6\) (680 \(\mu\)L). For 1-d \(^1\)H-NMR, 128 scans were collected, chemical shifts were calibrated to the solvent signal, and solvent peaks presaturated. Also, proton-proton total correlation spectroscopy (TOCSY), Nuclear Overhauser Effect Spectroscopy (NOSEY), \(^{13}\)C heteronuclear single quantum correlation spectroscopy (HSQC), and \(^{15}\)N-HSQC were 2-d experiments used to determine the proton assignments for the HBP1.
4.2.3.3. GPC and HPLC

A Waters 2690 separations module with a Waters 410 refractive index detector and a Wyatt MiniDAWN light scattering detector was used for the molecular weight (\(M_n\) and \(M_w\)) determination of PNVF. 100\(\mu\)L of a 1 mg/mL PNVF solution was loaded onto a Ranin Separon HEMA-BIO 100 (8 mm \(\times\) 250 mm) size exclusion column at 1 mL/min. and developed with ultrapure water at the same flow rate. The Astra, version 4.0, software aided in the molecular weight determination, and for this determination, PNVF was assumed to have a dn/dc constant of 0.17.

For reversed-phase HPLC purification of PEO-HBP1, a Waters 2690 separations module was used along with a Waters 2487 UV absorbance detector controlled by the Millennium software (v. 2.0). Peptide (less than 100 mg) was dissolved in no more than 2 mL of the mobile phase, 0.1% TFA in ultra pure water, and loaded onto an Alltech Hyperprep PEP 100Å 8u (22 mm \(\times\) 250 mm) preparatory scale C-18 column. A 26% - 41% acetonitrile/0.082% TFA gradient against the mobile phase was run over 45 minutes [4.37]. UV absorbance was monitored at 220 nm and 280 nm, wavelengths specific for the peptide bond and the tryptophan residue, respectively.

4.2.3.4. Mass Spectrometry

A PerSeptive Biosystems matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) with a Voyager Biospectrometry Workstation was used to provide molecular weight analysis of PEO-HBP. Also used was a Bruker
Biflex III MALDI-MS. Analysis via this sensitive technique, which has a mass accuracy between ± 0.01% and ± 0.1%, is useful to distinguish among the desired peptide and its unwanted derivatives. Less than 0.1 mg of the sample was dissolved in ultra pure water (1 mL). The matrix, α-cyano-4-hydroxycinnamic acid, was dissolved at 25 mg/mL in acetone. The peptide solution (1 μL) and the matrix solution (1 μL) were added together to make a small drop on the metal target plate. 200 scans were collected over a range of 800 m/z – 3600 m/z.

4.2.3.5. Molecular Modeling of Peptide Surfactant Polymer

Molecular modeling analysis was performed with the aid of Insight II (MSI, Inc.) molecular modeling software running on an Octane2 workstation (Silicon Graphics Inc.). First, the molecular coordinates for the crystal structure of fibronectin were downloaded from the Brookhaven Protein Data Bank, and the HBP1, HBP2, and RGD relevant fragments were extracted and modified to resemble HBP1, HBP2, and RGD respectively [4.38]. This structure was then relaxed using an energy minimization calculation that accounts for solvation energy (Discover module, derivative, force field). Understanding of the ligand’s molecular dimensions was drawn from this final conformation.
4.2.4. Preparation of Surfaces

4.2.4.1. Octadecyltrichlorosilane Treated Glass

Octadecyltrichlorosilane (OTS) modified glass cover slips were used as a model hydrophobic substrate. This material was also chosen because it is manageable, smooth, and optically transparent, a necessity for the microscopic investigation of endothelial cell viability. The preparation of these surfaces was adapted from a well-accepted method [4.39]. Glass cover slips were sonicated for 30 minutes in HPLC grade chloroform followed by rinsing with a stream of chloroform. After drying, the discs, not allowed to overlap, were placed in three small glass petri dish covers and subjected to radio frequency induced argon and water vapor plasma by a Branson plasma cleaner (500 mTorr, 50 W forward power, 0 W reflected power). Following plasma cleaning on both sides, the discs were immersed for 30 minutes on each side in a fresh solution of 100 µL of OTS in 10 mL dicyclohexyl, enough to modify 30 cover slips. The OTS modified cover slips were then cleaned with 3 more sonication and rinsing cycles. The resulting material yielded a water contact angle between 100 and 110 degrees.

4.2.4.2. Surfactant Adsorbed Surfaces

The surfactant polymers were adsorbed onto the OTS-modified cover slips by immersing the discs into a surfactant and ultra pure water solution. The solution (1 mg/mL) was sterile filtered and dispensed into sterile vials, and the discs had been
sterilized via ethylene oxide gas sterilization (Cleveland Clinic, Cleveland, OH). After static adsorption for 24 hours, the solution was diluted by three exchanges with ultra pure water so that little surfactant would become loosely bound after pulling a disc through the surfactant-concentrated air/water interface.

4.2.4.3. Fibronectin Coated Glass

Fibronectin (FN) from rabbit plasma was affinity purified by sequential use of gelatin agarose and heparin. Agarose chromatography was performed as described by Miekka et al. in which the FN is eluted with 3 M urea, the reagent is quickly removed by absorption onto heparin-agarose, followed by 0.4 M NaCl elution [4.40].

15 mm circular glass cover slips were disinfected in 100% ethanol. They were then sterilized via a cold (room temperature) ethylene oxide sterilization process with a 24 h outgassing time. In order to coat the glass surfaces with FN (1 μg/cm²), rabbit fibronectin was suspended in PBS. The amount of PBS needed was calculated by multiplying the number of cover slips to be coated by 400 μL (the volume which prevented liquid from traveling past the boundaries of the glass cover slip). The amount of FN was determined by calculating the total surface area of all the cover slips to be coated. The PBS/FN solution was sterile filtered and a 400 μL aliquot PBS/FN mixture was placed onto the cleaned glass surface and allowed to adsorb for one hour at room temperature yielding 1 μg/cm² on each cover slip. This fibronectin-coated glass was used as a positive control for endothelial cell adhesion and growth.
4.2.5. Characterization of Surfactant Adsorbed Surfaces

4.2.5.1. Contact Angle

Water contact angle measurements were taken by the sessile drop method with the aid of a Rame Heart (model 100-00) goniometer via the sessile drop method. All samples were allowed to air dry for 24 hours before taking measurements. Advancing and receding measurements were taken by adding five 2 µL drops followed by withdrawing 2 µL at a time from the bead and taking a reading after each change.

4.2.5.2. Endothelialization

_Harvesting Endothelial Cells (ECs):_ Human Pulmonary Artery Endothelial Cells (HPAEC) were harvested from healthy, excess pulmonary artery segments taken from donor hearts during cardiac transplantation according to a procedure described by Grafe et al. [4.41]. Pulmonary artery segments 1-2 cm in diameter and 2 cm in length were stored on ice in PBS with 10% w/v streptomycin (PBS-S). Then, the solution was exchanged with fresh PBS-S at 4°C and incubated at 4°C for 15-30 min., followed by longitudinal cutting of the segments and three rinses with PBS-S. The segments were then placed flat, luminal side facing upward, and the corners were supported, forming a trough into which 500 µL of PBS with 0.2% type II collagenase was poured. The solution was incubated for 5 min. and then placed into a 15 mL centrifuge tube containing 500 µL of FBS. The surface of the arterial segment was scraped gently to
remove the uppermost gelatinous disassociated cell layer that was then washed into the centrifuge tube with FBS. The surface was scraped two more times, and the scrapings were placed in a separate centrifuge tube with 10 mL FBS. The cell suspensions were centrifuged at 200 g for 15 min. The supernatant was aspirated, and ~1 mL of the supernatant was left in the tube in order to not disturb the cell pellet. The resulting cells were resuspended in 5 mL of complete media, and then transferred into two wells of a 6 well tissue culture plate precoated with rabbit FN (1 µg/cm²). The cells were allowed to attach for 1h, washed twice with PBS-S, and then cultured in complete growth media until confluent.

**EC Culture:** MCDB-131 was utilized as the basal endothelial cell media. 1.2 g of sodium bicarbonate was added to the media to buffer the pH. The pH of the solution was analyzed with a standard hydrogen electrode (Baxter) and adjusted to 7.4. The media solution was then sterile filtered through a 0.22 µm pore size cellulose acetate bottle top filter and was stored for up to three months at 4°C. Complete growth media was used to culture the EC. The media was made by adding 0.015% w/v ECGS, 0.009% heparin w/v, and 10% v/v FBS into the basal media. The complete media was stored at 4°C for up to one month.

Purified rabbit FN (1 µg/cm²) was used to precoat either T-75 or T-25 culture flasks. PBS was mixed with the desired amount of FN and then adsorbed onto the surface at room temperature. After 1 h, the solution was aspirated, and either isolated ECs, passaged ECs, or frozen stores of ECs were resuspended in growth media and pipetted into the polystyrene culture flasks. Media was added to fully cover the flask bottom. The flask was placed into a 37°C tissue culture incubator (NuAire IR Autoflow
CO₂ water-jacketed Incubator) with 5% CO₂ and 70% relative humidity. The flask caps were left loose enough to allow for sufficient gas exchange. When the MCDB-131 media color started to change due to the phenol red pH indicator, which is red above pH 7 and yellow pH 7, the media was aspirated and replaced with fresh media. The cells were maintained in this manner until they reached approximately 85-95% confluence. Once this was reached, the cells were passaged.

**EC Passaging:** Flasks, which contained cells that were nearly confluent, were split at a 1:3 ratio into new flasks. Cell media was aspirated, and then the cells were rinsed with PBS. A 0.025% trypsin/0.01% EDTA solution in PBS was added to the flask, swirled gently, and quickly aspirated leaving a minimal amount in the flask. The flask was capped and tapped with the palm of the hand to “lift” the cells from the flask surface. The cells were then observed using phase contrast microscopy to ensure that they were beginning to round up and float about freely. The flask was then placed in the incubator for approximately 2 minutes to allow all of the cells to become mobile. The flask was again checked with the microscope to ensure that most of the cells had detached from the surface. If a significant amount of cells remained adherent, the flask was tapped again with the palm of the hand. Fresh complete growth media was placed into the flask and mixed thoroughly using an automatic pipette. The entire cell suspension was taken up in the graduated pipette and placed into a conical vial. The cells were then split and either cultured as described previously or stored for later use. Only EC under the 8th passage were used in the experiments.

**EC Storage:** Passaged EC were stored using the following procedure. The conical vial containing the cell suspension from the EC passaging procedure was centrifuged for 10
minutes at 4°C at 150 g. A cell pellet formed at the bottom of the tube, and the supernatant was aspirated from the vial. A desired amount of 10% DMSO in FBS was added to the vial and mixed gently to resuspend the cells. The cell suspension was transferred to sterile cryovials in 1 mL aliquots. These vials were stored at -70°C overnight and then transferred to a liquid nitrogen freezer for long term storage.

**EC Seeding:** EC that had grown to confluence were utilized to seed the test surfaces. The test materials were 15 mm circular cover slips each possessing an effective growth area of ~1.76 cm² allowing for a seeded cell density of approximately 5000 cells/cm². The number of cells/mL in suspension to be seeded was determined using a hemocytometer. A 50 μL aliquot of cell suspension was combined with 50 μL of 0.4% trypan blue and mixed thoroughly. Viable cells will absorb trypan blue, and dead cells will remain unstained, making distinction and counting easy. After adding the mixture to the hemocytometer, the cells settled and were then counted, and cell concentration was calculated. The remaining cell suspension was mixed again to resuspend cells, and media was added, if needed, to adjust the cell concentration to ensure the cover slips would be seeded at the desired cell density. The substrate materials were placed into separate wells of a culture plate and 400 μL of the cell suspension was dropped onto the cover slips, while not allowing any liquid to travel past the cover slip boundaries. The cells were left undisturbed for 1 h to allow them attachment, and then examined by phase contrast microscopy to verify the attachment. More of growth media (2 mL) was added to the wells, and the cells were then placed in an incubator maintained at 37°C and 5% atmospheric CO₂. The cells were allowed to grow to confluence (approximately 3 days), changing the media if necessary.
Microscopy of EC Cultured Surfaces: Prior to fixing, phase contrast images were taken of the cells on the different surfaces. Phase contrast microscopy images (not shown here) were acquired at 3, 6, 12, 24, and 48 hours using the Metamorph Imaging Software (Universal Imaging Corp.) package. Cells were then fixed at 3, 6, 12, 24, and 48 hours and blocked with 4% FBS in TBS at room temperature for 3 hours. Fixed cells were incubated for 1 hour in 1:1000 dilution of a mouse primary monoclonal antibody against vinculin (hVIN-1) in TBS with 4% FBS, washed with TBS, and incubated with a biotinylated anti-mouse IgG secondary antibody and streptavidin FITC (Excitation $\lambda = 494$ nm, Emission $\lambda = 518$ nm) in order to label the focal adhesion molecule vinculin. The cells were also stained with rhodamine phalloidin (Excitation $\lambda = 554$ nm, Emission $\lambda = 573$ nm) to label the actin stress fibers. Cells were analyzed with a Bio-Rad MRC 600 scanning confocal microscope. In the optical images, actin stress fibers show as red and focal adhesion molecule vinculin as green.

4.3. Results

4.3.1. Synthesis and Characterization of Peptide Surfactant Polymer

4.3.1.1. Poly(vinyl amine)

In the synthesis of the surfactant polymer, the PVAm backbone must first be synthesized and characterized. The synthesis of PVAm proceeded in three steps
described in Section 4.3.1 and shown in Scheme 4-1(A). In the first step, N-vinyl formamide was polymerized, and the resulting poly(N-vinyl formamide) was purified to yield a white powder (0.1733 g, a 24% yield). PNVF was analyzed by several methods to determine its composition, size and purity. An FT-IR spectrum showed absorptions consistent with PNVF as displayed in Table 4-1 and Figure 4-2. Small absorptions in the IR spectrum not indicated in Table 4-1 were attributed to the end groups of the polymer created either by the AIBN initiator, by chain transfer to the solvent, or by disproportionation. The $^1$H-NMR spectrum (Figure 4-3) in deuterium oxide showed three distinct peaks with the appropriate 2:1:1 integral ratio of $R_2$-$CH_2$ ($\delta$ 1.64 ppm), $R_3$-$CH$ ($\delta$ 3.90 ppm), and $R$-$CHO$ ($\delta$ 7.99 ppm). GPC was used to determine the absolute molecular weight by refractive index and light scattering detection, confirming that the polymer was well defined having an $M_n$ of 16,000 ± 6,000 g/mol and polydispersity of 1.5 ± 0.8 (See Figure 4-4).

In the second step, the formamide group of PNVF was hydrolyzed under basic conditions and purified to yield 0.1247 g (60% yield) of the yellow gummy aggregate of PVAm·HCl. Basic hydrolysis achieves a more complete conversion rather than acidic hydrolysis, which causes a build up of positive charges along the polymer thus inhibiting further formamide hydrolysis. Evidence of this complete conversion can be seen in the FT-IR (Figure 4-5 and Table 4-2) and $^1$H-NMR (Figure 4-6) spectra. The FT-IR spectrum showed very strong ammonium peaks along with no evidence of the Amide I peak at 1661 cm$^{-1}$. The additional peaks, assigned in Table 4-2, were consistent with a PVAm·HCl polymer. Also, the NMR spectra showed no evidence of a formamide proton
Figure 4-2. KBr pellet FT-IR spectra for PNVF. The IR absorption assignments are given in Table 4-1 and are consistent with a PNVF polymer. Relative absorbances not assigned in Table 4-1 were attributed to the polymer end groups.

Table 4-1. IR absorption assignments for PNVF.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3279</td>
<td>$\nu$(N-H), trans</td>
</tr>
<tr>
<td>3051</td>
<td>$\nu$(N-H), cis</td>
</tr>
<tr>
<td>2920</td>
<td>$\nu_a$(C-H) in CH₂</td>
</tr>
<tr>
<td>2865</td>
<td>$\nu_s$(C-H) in CH₂</td>
</tr>
<tr>
<td>2760</td>
<td>$\nu$(C-H) in CHO</td>
</tr>
<tr>
<td>1661</td>
<td>Amide I, $\nu$(C=O)</td>
</tr>
<tr>
<td>1533</td>
<td>Amide II, $\nu$(C-N) + $\delta$(N-H), trans</td>
</tr>
<tr>
<td>1441</td>
<td>$\delta$(C-H) in CH₂ and $\delta$(N-H), cis</td>
</tr>
<tr>
<td>1387</td>
<td>$\delta$(C-H) in CHO</td>
</tr>
<tr>
<td>1313</td>
<td>$\nu$(C-N), cis</td>
</tr>
<tr>
<td>1248</td>
<td>Amide III, $\nu$(C-N) + $\delta$(N-H)</td>
</tr>
<tr>
<td>761</td>
<td>$\rho$(CH₂)</td>
</tr>
</tbody>
</table>

$\nu$ = stretching, $\nu_s$ = symmetric stretching, $\nu_a$ = asymmetric stretching, $\delta$ = deformation, $\rho$ = rocking
Figure 4-3. 600 MHz $^1$H-NMR spectrum of PNVF in deuterium oxide. The peaks were assigned as (a), (b), and (c) with the inset chemical drawing. (a) indicates the CH$_2$ protons, (b) indicates the R$_3$-CH protons, and (c) indicates the CHO protons. The three peaks had the appropriate integral ratio of 2:1:1.
Figure 4-4. Results of absolute molecular weight determination using HPLC and the Astra software illustrating the well-defined structure of PNVF. (a) Chromatogram from the refractive index detector showing one narrow peak. (b) Plot showing molecular weight distribution of PNVF. (c) Plot showing the size distribution of the polymer. (d) Plot illustrating the relationship between molecular weight and radius.
Figure 4-5. KBr pellet FT-IR spectra for PVAm•HCl. The IR absorption assignments are given in Tables 4-2 and are consistent with a PVAm•HCl polymer. There was no longer an amide I peak at 1661 cm⁻¹ from the formamide group of PNVF; a primary amine deformation absorbance at 1607 cm⁻¹ now exists. Relative absorbances not assigned in Table 4-2 were attributed to the polymer end groups.

Table 4-2. IR absorption assignments for PVAm•HCl.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3425</td>
<td>ν(N-H)</td>
</tr>
<tr>
<td>2948</td>
<td>νₐ(C-H) and νₛ(C-H)</td>
</tr>
<tr>
<td>2555</td>
<td>ν(N-H), sh.</td>
</tr>
<tr>
<td>2004</td>
<td>combination δ(N-H) + NH₃ torsion</td>
</tr>
<tr>
<td>1607</td>
<td>δₐ(N-H)</td>
</tr>
<tr>
<td>1506</td>
<td>δₛ(N-H) and δ(C-H)</td>
</tr>
<tr>
<td>1168</td>
<td>δ(C-C-N)</td>
</tr>
<tr>
<td>1021</td>
<td>ν(C-N)</td>
</tr>
</tbody>
</table>

ν = stretching, νₛ = symmetric stretching, νₐ = asymmetric stretching, δ = deformation, ρ = rocking
Figure 4-6. 600 MHz $^1$H-NMR spectrum of PVAm·HCl in deuterium oxide. The peaks were assigned as (a) and (b) with the inset chemical drawing. (a) indicates the CH$_2$ protons and (b) indicates the R$_3$-CH protons. These two peaks had the appropriate integral ratio of 2:1, and no peaks were observed for the amine protons due to proton exchange with the solvent. Also, there is no longer a peak at 7.99 ppm reinforcing that complete hydrolysis of the formamide groups had taken place.
at 7.99 ppm, but displayed two peaks due to protons along the polymer backbone with the appropriate 2:1 integral ratio of R₂-CH₂ (δ 2.00 ppm) and R₃-CH (δ 3.58 ppm).

In the third step, strong anion exchange chromatography was used to convert PVAm·HCl to PVAm, and to purify the polymer, resulting in 24 mg (a 44% yield) of a white powder. An IR spectrum of PVAm (Figure 4-7 with peak assignments in Table 4-3) was almost identical to PVAm·HCl. However, the multiple bands in the region of 2000 – 2600 cm⁻¹, a characteristic of –NH₃⁺ functional groups [4.42], were absent, hinting that the conversion from amine hydrochloride to amine was completed. Stronger evidence of this conversion was provided in the NMR spectrum (Figure 4-8). The methylene protons and methine protons shifted upfield by approximately 0.6 ppm to 1.37 ppm and 2.98 ppm, respectively. This indicates that these protons are in the vicinity of an amine which had less of a deshielding effect compared to that of an amine salt [4.43]. The IR and NMR characterization showed that well-defined PVAm was prepared.

4.3.1.2. Peptide Ligands

HBP1 was made by solid phase peptide synthesis and characterized by reversed-phase HPLC, mass spectrometry, FT-IR, NMR, and fluorescence. Each step of the solid phase peptide synthesis appeared to be sufficient because the synthesizer’s conductivity monitor showed no step with a markedly lower level of charged species, which are interpreted as byproducts of deprotection. A smaller scale cleavage was performed to yield a small amount of the peptide for analysis. To purify, reversed-phase HPLC was used to separate HBP1 from incomplete cleavage products (Figure 4-9). (A 26% - 41%
Evidence of the existence of an amine as opposed to an amine salt can be seen in the lack of multiple bands in the 2000 - 2600 cm⁻¹ region, which is a characteristic of the salt. The IR absorption assignments are given in Tables 3-3 and are consistent with those of a PVAm polymer. Relative absorptions not assigned in Table 4-3 were attributed to the polymer end groups.

**Figure 4-7.** KBr pellet FT-IR spectra for PVAm. Evidence of the existence of an amine as opposed to an amine salt can be seen in the lack of multiple bands in the 2000 - 2600 cm⁻¹ region, which is a characteristic of the salt. The IR absorption assignments are given in Tables 3-3 and are consistent with those of a PVAm polymer. Relative absorptions not assigned in Table 4-3 were attributed to the polymer end groups.

**Table 4-3.** IR absorption assignments for PVAm.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3353</td>
<td>νa(N-H) and νs(N-H)</td>
</tr>
<tr>
<td>2930</td>
<td>νa(C-H) and νs(C-H)</td>
</tr>
<tr>
<td>1585</td>
<td>δ(N-H)</td>
</tr>
<tr>
<td>1448</td>
<td>δ(CH₂)</td>
</tr>
<tr>
<td>1332</td>
<td>δ(CH)</td>
</tr>
<tr>
<td>1146</td>
<td>ν(C-N)</td>
</tr>
<tr>
<td>820</td>
<td>ρ(NH₂)</td>
</tr>
<tr>
<td>727</td>
<td>ρ(CH₂)</td>
</tr>
</tbody>
</table>

ν = stretching, νa = symmetric stretching, νs = asymmetric stretching, δ = deformation, ρ = rocking
Figure 4-8. 600 MHz $^1$H-NMR spectrum of PVAm in deuterium oxide. The peaks were assigned as (a) and (b) with the inset chemical drawing. (a) indicates the CH$_2$ protons, and (b) indicates the R$_3$-CH protons. These two peaks also had the appropriate integral ratio of 2:1. No peak was observed for the amine protons in the deuterium oxide spectrum due to proton exchange with the solvent. Evidence of the conversion from PVAm·HCl to PVAm can be seen in the upfield shift (~0.6 ppm) of both peaks. Protons in the vicinity of the amine were more shielded than those in the vicinity of the amine salt. However, if exchange is rapid with respect to the NMR time scale, then these signals are averages, and the upfield shift of these average signals indicates an increased amount of the amine compared to the salt.
Figure 4-9. Reversed-phase HPLC purification of HBP1. The UV chromatogram at a wavelength of 220 nm shows species with a peptide bond and the 280 nm wavelength is specific for the tryptophan residue. The first large peak, eluting from 16 - 27 minutes, was collected, and the other later peaks were assumed to be the unwanted by products of either the peptide synthesis or the cleavage reaction.
acetonitrile/0.082% TFA gradient against the mobile phase was run over 45 minutes.)
The fraction between 15 and 20 minutes was collected. MALDI-TOF mass spectrometry
was used to verify that pure HBP1 was collected. In Figure 4-10, there is one large peak
corresponding to HBP1 ([M + H] = 1399 (measured), 1397.74 (calculated)). All other
peaks were considered relatively insignificant. Most importantly, there were no peaks
above [M + H], corresponding to any remaining protecting groups (+Trt = [M + 243],
+Pbf = [M + 253], +tBu = [M + 57], and +Fmoc = [M + 223]), and there were no peaks
below [M + H], which would correspond to amino acid deletions (-A = [M - 71], -G =
[M - 57], -I = [M - 113], -P = [M - 97], -Q = [M - 128], -R = [M - 156], -S = [M - 87],
and -W = [M - 186]).

An FT-IR spectrum of a KBr pellet of HBP1 (shown in Figure 4-11 with peak
assignments in Table 4-4) was taken in order to establish a background for future
modifications, as in the syntheses of PEO-HBP1 and PVAm(HBP1:Hex). Dominated by
N—H and O—H stretches and the Amide I carbonyl stretch, the spectrum was very
typical of any peptide. The composition of the purified peptide was also verified using
600 MHz NMR. (The labeled peaks of the 1-d spectrum are shown in Figure 4-12, and
the labeled peaks of useful 2-d spectra, which aided in the peak identification of the 1-d
spectrum, are shown in Figure 4-13.)

In order to support the hypothesis that the PVAm(HBP1:Hex) polymer surfactant
encourages endothelial cell attachment through interaction with cell surface heparan
sulfate proteoglycans, it must be shown that HBP1 interacts with heparan sulfate or
heparin. To detect an interaction, the fluorescence emission, measured in counts per
seconds (cps), of HBP1 alone and in the presence of heparin was compared. A 10.0 μM
Figure 4-10. MALDI-TOF mass spectrum of purified HBP1. The most prominent peak at 1399 corresponds to HBP1, or $[\text{M} + \text{H}]^+$. Other peaks were considered insignificant in comparison because of their low signal.
Figure 4-11. KBr pellet FT-IR spectra for HBP1. The spectrum was very typical of a peptide with the absorption assignments listed in Table 4-4. This spectrum established a background in order to verify future synthetic modifications.

Table 4-4. IR absorption assignments for HBP1.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3346</td>
<td>ν(N-H), trans; ν(O-H); ν(N-H) amine</td>
</tr>
<tr>
<td>3214</td>
<td>ν(N-H), cis</td>
</tr>
<tr>
<td>3043</td>
<td>ν(C-H) in Trp aromatic ring</td>
</tr>
<tr>
<td>2971</td>
<td>νₐ(C-H) in CH₃</td>
</tr>
<tr>
<td>2943</td>
<td>νₐ(C-H) in CH₂</td>
</tr>
<tr>
<td>2884</td>
<td>νₐ(C-H) in CH₃, CH₂; ν(C-H) in CHR₃</td>
</tr>
<tr>
<td>1671</td>
<td>Amide I, ν(C=O)</td>
</tr>
<tr>
<td>1537</td>
<td>Amide II, ν(C-N) + δ(N-H)</td>
</tr>
<tr>
<td>1442</td>
<td>δ(N-H); δ(C-C); in-plane aromatic ring</td>
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<td>Amide III, ν(C-N) + δ(N-H)</td>
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<td>1189</td>
<td>ν(C-N); in-plane δ(C-H) aromatic ring</td>
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<td>1137</td>
<td>ν(C-O); in-plane δ(C-H) aromatic ring</td>
</tr>
<tr>
<td>1074</td>
<td>in-plane δ(C-H) aromatic ring</td>
</tr>
<tr>
<td>929</td>
<td>out-of-plane δ(C-H) aromatic ring</td>
</tr>
<tr>
<td>840</td>
<td>out-of-plane δ(C-H) aromatic ring</td>
</tr>
<tr>
<td>802</td>
<td>wag(N-H)</td>
</tr>
<tr>
<td>749</td>
<td>out-of-plane δ(C-H) aromatic ring</td>
</tr>
<tr>
<td>723</td>
<td>ρ(CH₂)</td>
</tr>
</tbody>
</table>

ν = stretching, νₛ = symmetric stretching, νₐ = asymmetric stretching, δ = deformation, ρ = rocking
Figure 4-12. 600 MHz $^1$H-NMR spectrum of HBP1 in d$_6$-DMSO. The peaks were assigned according to the labeled amino acid residues of the inset HBP1 chemical drawing.
Figure 4-13. 2d-NMR spectra of HBP1 in d$_6$-DMSO. These 2-d spectra aided in the proton assignments shown in Figure 3-11. (a) An $^{15}$N-HSQC 2-d spectrum, detecting proton-nitrogen couplings. In particular, notice the two amide groups at 109.5 ppm and 111.0 ppm, where two protons attached to the same nitrogen have different proton shifts. Also, there were no peaks detected for the amide terminus and the $R_\eta$ guanido groups, due to the broadness of these peaks. (b) A $^{13}$C-HSQC spectrum aided in distinguishing magnetically different protons bonded to the same carbon as occurs in $S_\beta$, $W_{\beta}$, $Q_\beta$, $Q_\gamma$, $P_\beta$, $P_\gamma$, $P_\delta$, $R_\beta$, $R_\gamma$, $R_\delta$, and $I_\gamma$. Also shown are two helpful sections of a TOCSY spectrum: (c) The correlation peaks between the amide and alpha protons and (d) the correlation peaks between the alpha protons of an amino acid and its corresponding $\beta$, $\gamma$, and $\delta$ protons.
A peptide solution excited at 280 nm had a fluorescence emission of 573,000 cps at 362 nm. A 1.0 μM solution of heparin excited at 280 nm had a fluorescence emission of 50,000 cps at 362 nm. It was not expected that the heparin would fluoresce in this region, but there could be residual protein impurities since the heparin sample originated from porcine intestinal mucosa. The heparin sample’s fluorescence was unfortunate since it prevented generating a useful fluorescence titration curve for measurement of a dissociation constant, K_d. However, a qualitative conclusion of interaction was drawn when the fluorescence of a 10 μM HBP1 and 1 μM heparin mixture was measured to be 504,000 cps. Due to the linear nature of fluorescence at dilute concentrations and if there was no interaction between HBP1 and the heparin, the expected measured fluorescence would equal the sum of the previous two measurements or 623,000 cps, not 504,000 cps. However, interaction between the two molecules was inferred from the nonlinear decrease in fluorescence resulting from changes in the tryptophan environment. As a result, HBP1 was synthesized, characterized, and found to interact to some degree with heparin in solution.

HBP2 was made by solid phase peptide synthesis and characterized by reversed-phase HPLC, mass spectrometry, and FT-IR, NMR. Each step of the solid phase peptide synthesis appeared to be sufficient because the synthesizer’s conductivity monitor showed no step with a markedly lower level of charged species, which are interpreted as byproducts of deprotection. A smaller scale cleavage was performed to yield a small amount of the peptide for analysis. To purify, reversed-phase HPLC was used to separate HBP2 from incomplete cleavage products (Figure 4-14). (A 25% - 40% acetonitrile/0.082% TFA gradient against the mobile phase was run over 45 minutes.)
Figure 4-14. Reversed-phase HPLC purification of HBP2. The UV chromatogram at a wavelength of 220 nm shows species with a peptide bond and the 280 nm wavelength is specific for the tryptophan residue. The first large peak, eluting from 14 - 18 minutes, was collected, and the other later peaks were assumed to be the unwanted byproducts of either the peptide synthesis or the cleavage reaction.
The fraction between 14 and 18 minutes was collected. MALDI-TOF mass spectrometry was used to verify that pure HBP2 was collected. In Figure 4-15, there is one large peak corresponding to HBP2 ([M + H] = 1513.52 (measured), 1512.81 (calculated)). All other peaks were considered relatively insignificant. Most importantly, there were no peaks above [M + H], corresponding to any remaining protecting groups (+Pbf = [M + 253], +tBu = [M + 57], and +Fmoc = [M + 223]), and there were no peaks below [M + H], which would correspond to amino acid deletions (-A = [M - 71], -G = [M - 57], -V = [M - 99], -P = [M - 97], -R = [M - 156], -S = [M - 87], -T = [M - 101], and -W = [M - 186]).

An FT-IR spectrum of a KBr pellet of HBP2 (shown in Figure 4-16 with peak assignments in Table 4-5) was taken in order to establish a background for future modifications, as in the syntheses of PEO-HBP2 and PVAm(HBP2:Hex). Dominated by N—H and O—H stretches and the Amide I carbonyl stretch, the spectrum was very typical of any peptide. The composition of the purified peptide was also verified using 600 MHz NMR. (The labeled peaks of the 1-d spectrum are shown in Figure 4-17.)

The analysis of an RGD peptide prior to the coupling of a spacer is contained in Chapter 3 (section 3.4.1). The mass spectrum of the RGD peptide is shown in Figure 4-18. The most prominent peak (1076 measured, 1075.49 calculated) is the desired peptide, or RGD ([M + H]⁺). There were also peaks corresponding to [M + Na]⁺ and [M + K]⁺. The peak at 1133 corresponds to an insignificant quantity of RGD still containing one tert-butyl protecting group, [M + 58]⁺. The peaks at 1328, 1350, and 1366 correspond to the [M + Pbf + H]⁺, [M + Pbf + Na]⁺, and [M + Pbf + K]⁺, respectively. These peaks result from the incomplete cleavage of the Pbf protecting group from a small amount of arginine residues. The amount of this species is also relatively insignificant
Figure 4-15. MALDI-TOF mass spectrum of purified HBP1. The most prominent peak at 1513 corresponds to HBP2, or [M + H]^+. The peak at [M + 23]^+ corresponds to the [M + Na]^+. Other peaks were considered insignificant in comparison because of their low signal.
Figure 4-16. KBr pellet FT-IR spectra for HBP2. The spectrum was very typical of a peptide with the absorption assignments listed in Table 4-4b. This spectrum established a background in order to verify future synthetic modifications.
### Table 4-5. IR absorption assignments for HBP2.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3403 - 3268</td>
<td>(\nu)(N-H), trans; (\nu)(O-H); (\nu)(N-H), amine</td>
</tr>
<tr>
<td>3221</td>
<td>(\nu)(N-H), cis</td>
</tr>
<tr>
<td>3068</td>
<td>(\nu)(C-H) in Trp aromatic ring</td>
</tr>
<tr>
<td>2971</td>
<td>(\nu_a)(C-H) in CH(_3)</td>
</tr>
<tr>
<td>2933</td>
<td>(\nu_a)(C-H) in CH(_2)</td>
</tr>
<tr>
<td>2889 - 2875</td>
<td>(\nu_a)(C-H) in CH(_3) and CH(_2); (\nu)(C-H) in CHR(_3)</td>
</tr>
<tr>
<td>1667</td>
<td>Amide I, (\nu)(C=O)</td>
</tr>
<tr>
<td>1534</td>
<td>Amide II, (\nu)(C-N) + (\delta)(N-H)</td>
</tr>
<tr>
<td>1453</td>
<td>(\delta)(N-H); (\delta)(C-C); in-plane aromatic ring</td>
</tr>
<tr>
<td>1379</td>
<td>(\delta)(CH(_3))</td>
</tr>
<tr>
<td>1341</td>
<td>(\delta)(O-H)</td>
</tr>
<tr>
<td>1273</td>
<td>in-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>1239</td>
<td>Amide III, (\nu)(C-N) + (\delta)(N-H)</td>
</tr>
<tr>
<td>1204 - 1184</td>
<td>Aliphatic skeletal vibration</td>
</tr>
<tr>
<td>1136</td>
<td>(\nu)(C-O); in-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>1076</td>
<td>(\nu)(C-N) amine; in-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>930</td>
<td>out-of-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>837</td>
<td>out-of-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>802</td>
<td>wag(N-H)</td>
</tr>
<tr>
<td>747</td>
<td>out-of-plane (\delta)(C-H) aromatic ring</td>
</tr>
<tr>
<td>722</td>
<td>(\rho)(CH(_2))</td>
</tr>
</tbody>
</table>

\(\nu\) = stretching, \(\nu_s\) = symmetric stretching, \(\nu_a\) = asymmetric stretching, 
\(\delta\) = deformation, \(\rho\) = rocking
**Figure 4-17.** 600 MHz $^1$H-NMR spectrum of HBP2 in d$_6$-DMSO. The peaks were assigned according to the labeled amino acid residues of the inset HBP2 chemical drawing.
Figure 4-18. MALDI-TOF mass spectrum of purified RGD. The most prominent peak at 1076 corresponds to RGD, or [M + H]⁺. The peak at [M + 23]⁺ corresponds to the [M + Na]⁺. The peak at [M + 39]⁺ corresponds to the [M + K]⁺. The peak at 1328 corresponds to the [M + Pbf + H]⁺. Also, evident in the spectrum are the [M + Pbf + Na]⁺ and [M + Pbf + K]⁺ peaks. The peak at 1133 corresponds to an insignificant quantity of [M + tBu]⁺. Other peaks were considered insignificant in comparison because of their low signal, and the peaks at 1037 and 1679 are impurities unrelated to the synthesized peptide.
because MALDI-TOF greatly over exaggerates peptides still containing a Pbf group [4.44]. Other peaks were considered insignificant in comparison because of their low signal, and the peaks at 1037 and 1679 are impurities unrelated to the synthesized peptide. The peptides xHBP1 and xHBP2 were not cleaved, deprotected, nor analyzed prior to PEO coupling.

4.3.1.3. PEO-peptides

4.3.1.3.1. HBP1-PEO

In order to enable the coupling of HBP1 to PVAm, the amino terminus of HBP1 was modified with 3,6,9-trioxaundecanedioic acid to create an N-terminal acid. After cleavage of the product, it was purified by reversed-phase HPLC, whose chromatogram is shown in Figure 4-19. The HBP1-PEO, which eluted from 13 min. to 18 min., was effectively separated from other impurities, shown by the other peaks. MALDI-MS (Figure 4-20), FT-IR (Figure 4-21), and $^1$H-NMR (Figure 4-22) were used to verify sample composition and purity. The mass spectrum of HBP1-PEO showed the most prominent peak at 1603, corresponding to HBP1-PEO (1602 calculated). There exist the usual peaks corresponding to HBP1-PEO with Na$^+$ and with K$^+$. There was a small amount of impurity at 1855, corresponding to a Pbf protecting group still attached to one arginine. Also noticeable were peaks containing PEO’s of differing degrees of polymerization—1559, -1PEO; 1515, -2PEO; 1427, -4PEO. There was a barely perceptible peak at 2984 corresponding to the dimer, HBP1-PEO-HBP1. This dimer
Figure 4-19. Reversed-phase HPLC purification of HBP1-PEO. The UV chromatogram at a wavelength of 210 nm shows all species with any carbonyl group, particularly the peptide bonds, and the 280 nm wavelength is specific for the tryptophan residue. A fraction eluting from 13 – 18 min. was collected as HBP1-PEO.
Figure 4-20. MALDI-TOF mass spectrum of HBP1-PEO. The most prominent peak at 1603 corresponds to HBP1-PEO, or \([M + H]^+\). There do exist the usual peaks corresponding to \([M + Na]^+\) and \([M + K]^+\). The peak at 1855 is a small amount of the impurity, \([M + Pbf + H]^+\). Also noticeable are peaks containing PEO’s of differing degrees of polymerization—1559, \([M - PEO + H]^+\); 1515, \([M - 2PEO + H]^+\); 1427, \([M - 4PEO + H]^+\). Other peaks were considered insignificant in comparison because of their low signal. Also noteworthy is the absence of HBP1 at 1398.
Figure 4-21. KBr pellet FT-IR spectrum for HBP1-PEO. The spectrum closely resembled that of HBP1 except for a few absorbances due to the presence of PEO.

Table 4-6. IR absorption assignments for HBP1-PEO not attributed to HBP1.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800 - 3000</td>
<td>Increased relative absorption in (\nu(\text{C-H})) region</td>
</tr>
<tr>
<td>1408</td>
<td>(\delta(\text{O-H})), acid</td>
</tr>
<tr>
<td>1251</td>
<td>(\nu(\text{C-O})), acid</td>
</tr>
<tr>
<td>1065</td>
<td>(\nu_a(\text{C-O-C})), ether</td>
</tr>
<tr>
<td>892</td>
<td>out-of-plane (\delta(\text{O-H})), acid; (\nu_a(\text{C-O-C})), ether</td>
</tr>
</tbody>
</table>

\(\nu = \text{stretching}, \nu_s = \text{symmetric stretching}, \nu_a = \text{asymmetric stretching}, \delta = \text{deformation}, \rho = \text{rocking}\)
Figure 4-22. NMR spectrum of HBP1-PEO in d$_6$-DMSO. The downfield segment of the spectrum shows the acid proton at 12.6 ppm, indicating that the N-terminal modification of HBP1 was successful. Also, at 6.95 ppm there is a new triplet peak of the N-terminal glycine amide proton. The ethylene oxide protons are a broad peak at 3.4 ppm.
peak and other peaks were considered insignificant in comparison because of their low signal. Also noteworthy is the absence of HBP1 at 1398.

The FT-IR spectrum of HBP1-PEO (Figure 4-21) resembled very closely that of HBP1. Since there was only a small N-terminal modification, evidence of the PEO conjugation was relegated to a few peaks as shown in Table 4-6. There was an increase in the relative absorbance in the aliphatic C-H stretching region of the spectrum (2800 - 3000 cm⁻¹). There are new peaks due to the presence of an acid functionality at 1408, 1250, and 895 cm⁻¹. Most conspicuous of all is the C-O-C stretch from PEO at 1065 cm⁻¹.

The NMR spectrum of HBP1-PEO (Figure 4-22) also closely resembled that of HBP1; however, there were a few differences. The downfield segment of the spectrum shows the acid proton at ~12.6 ppm, indicating that the N-terminal modification of HBP1 was successful. Also, at 6.95 ppm there is a new triplet peak of the N-terminal glycine amide proton. The ethylene oxide protons appear as a broad peak at 3.4 ppm.

4.3.1.3.2. HBP2-PEO

In order to enable the coupling of HBP2 to PVAm, the amino terminus of HBP2 was modified with 3,6,9-trioxaundecanedioic acid to create an N-terminal acid. After cleavage of the product, it was purified by reversed-phase HPLC, whose chromatogram is shown in Figure 4-23. The HBP2-PEO, which eluted from 15 min. to 20 min., was effectively separated from other impurities, shown by the other peaks. MALDI-MS (Figure 4-24), FT-IR (Figure 4-25), and ¹H-NMR (Figure 4-26) were used to verify
**Figure 4-23.** Reversed-phase HPLC purification of HBP2-PEO. The UV chromatogram at a wavelength of 210 nm shows all species with any carbonyl group, particularly the peptide bonds, and the 280 nm wavelength is specific for the tryptophan residue. A fraction eluting from 15 – 20 min. was collected as HBP2-PEO.
Figure 4-24. MALDI-TOF mass spectrum of HBP2-PEO, showing the most prominent peak at 1718 (HBP2-PEO, 1717 calculated). There was a small amount of impurity at 1970, corresponding to a Pbf protecting group still attached to one arginine. There was also a peak at 3211 corresponding to a small amount of dimer, HBP2-PEO-HBP2. Other peaks were considered insignificant in comparison because of their low signal. Also noteworthy is the absence of HBP2 at 1513.
Figure 4-25. KBr pellet FT-IR spectrum for HBP2-PEO. The spectrum closely resembled that of HBP2 except for a few absorbances due to the presence of PEO.

Table 4-7. IR absorption assignments for HBP1-PEO not attributed to HBP2.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800 - 3000</td>
<td>Increased relative absorption in ν(C-H) region</td>
</tr>
<tr>
<td>1408</td>
<td>δ(O-H), acid</td>
</tr>
<tr>
<td>1250</td>
<td>ν(C-O), acid</td>
</tr>
<tr>
<td>1065</td>
<td>νₐ(C-O-C), ether</td>
</tr>
<tr>
<td>895</td>
<td>out-of-plane δ(O-H), acid; νₐ(C-O-C), ether</td>
</tr>
</tbody>
</table>

ν = stretching, νₛ = symmetric stretching, νₐ = asymmetric stretching, δ = deformation, ρ = rocking
Figure 4-26. NMR spectrum of HBP2-PEO in $d_6$-DMSO. The downfield segment of the spectrum shows the acid proton at 12.6 ppm, indicating that the N-terminal modification of HBP2 was successful. The ethylene glycol protons are a broad peak at 3.4 ppm.
sample composition and purity. The mass spectrum of HBP2-PEO showed the most prominent peak at 1718, corresponding to HBP2-PEO (1717 calculated). There was a small amount of impurity at 1970, corresponding to a Pbf protecting group still attached to one arginine. There was also a peak at 3211 corresponding to a small amount of dimer, HBP2-PEO-HBP2. Other peaks were considered insignificant in comparison because of their low signal. Also noteworthy is the absence of HBP2 at 1513.

The FT-IR spectrum of HBP2-PEO (Figure 4-25) resembled very closely that of HBP2. Since there was only a small N-terminal modification, evidence of the PEO conjugation was relegated to a few peaks as shown in Table 4-7. There was an increase in the relative absorbance in the aliphatic C-H stretching region of the spectrum (2800 - 3000 cm⁻¹). There are new peaks due to the presence of an acid functionality at 1408, 1250, and 895 cm⁻¹. Most conspicuous of all is the C-O-C stretch from PEO at 1065 cm⁻¹.

The NMR spectrum of HBP2-PEO (Figure 4-26) also closely resembled that of HBP2; however, there were a few differences. The downfield segment of the spectrum shows the acid proton at ~12.6 ppm, indicating that the N-terminal modification of HBP2 was successful. The ethylene oxide protons appear as a broad peak at 3.4 ppm.

4.3.1.3.3. RGD-PEO

In order to enable the coupling of RGD to PVAm, the amino terminus of RGD was modified with 3,6,9-trioxaundecanedioic acid to create an N-terminal acid. After cleavage of the product, it was purified by reversed-phase HPLC, whose chromatogram is
shown in Figure 4-27. The RGD-PEO, which eluted from 21 min. to 26 min., was
effectively separated from other impurities, shown by the other peaks. MALDI-MS
(Figure 4-28), FT-IR (Figure 4-29), and $^1$H-NMR (Figure 4-30) were used to verify
sample composition and purity. The mass spectrum of RGD-PEO showed the most
prominent peak at 1280, corresponding to RGD-PEO (1279.49 calculated). Other peaks
were considered insignificant in comparison because of their low signal. Also
noteworthy is the absence of RGD at 1075.

The FT-IR spectrum of RGD-PEO (Figure 4-29) resembled very closely that of
RGD. Since there was only a small N-terminal modification, evidence of the PEO
conjugation was relegated to a few peaks as shown in Table 4-8. There was an increase
in the relative absorbance in the aliphatic C-H stretching region of the spectrum (2800 -
3000 cm$^{-1}$). There are new peaks due to the presence of an acid functionality at 1729,
1406, 1249, and 882 cm$^{-1}$. Most conspicuous of all is the C-O-C stretch from PEO at
1092 cm$^{-1}$.

The NMR spectrum of RGD-PEO (Figure 4-30) also closely resembled that of
RGD; however, there were a few differences. The downfield segment of the spectrum
shows the acid proton with increased intensity at ~12.5 ppm, indicating that the N-
terminal modification of RGD was successful. Also, there are now five triplet peaks
instead of four in the amide proton region. The fifth triplet is $N_\alpha$ of the N-terminal
glycine residue has changed from an amine to an amide. The ethylene oxide protons
appear as a broad peak at 3.5 ppm.
Figure 4-27. Reversed-phase HPLC purification of RGD-PEO. The UV chromatogram at a wavelength of 210 nm shows all species with any carbonyl group, particularly the peptide bonds, and the 280 nm wavelength is specific for the tryptophan residue. A fraction eluting from 21 – 26 min. was collected as RGD-PEO.
Figure 4-28. MALDI-TOF mass spectrum of RGD-PEO. The most prominent peak at 1280 corresponds to RGD-PEO, or [M + H]^+. The peak at 1532 is a small amount of the impurity, [M + Pbf + H]^+. Also noticeable are peaks containing PEO’s of differing degrees of polymerization—1236, [M – PEO + H]^+ and 1192, [M - 2PEO + H]^+. Other peaks were considered insignificant in comparison because of their low signal. The smaller inset spectrum is RGD-PEO before HPLC purification. Notice the higher signal for [M + Pbf + H]^+ and the incidence of the dimer, RGD-PEO-RGD. Also noteworthy in both spectra is the absence of RGD at 1075.
Figure 4-29. KBr pellet FT-IR spectrum for RGD-PEO. The spectrum closely resembled that of RGD.

Table 4-8. IR absorption assignments for RGD-PEO not attributed to RGD.

<table>
<thead>
<tr>
<th>Wavenumber (cm(^{-1}))</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800 - 3000</td>
<td>Increased relative absorption in v(C-H) region</td>
</tr>
<tr>
<td>1729</td>
<td>Increased relative absorption in v(C=O) region</td>
</tr>
<tr>
<td>1406</td>
<td>δ(O-H), acid</td>
</tr>
<tr>
<td>1249</td>
<td>v(C-O), acid</td>
</tr>
<tr>
<td>1092</td>
<td>v(_d)(C-O-C), ether</td>
</tr>
<tr>
<td>882</td>
<td>out-of-plane δ(O-H), acid</td>
</tr>
</tbody>
</table>

v = stretching, v\(_s\) = symmetric stretching, v\(_a\) = asymmetric stretching, 
δ = deformation, ρ = rocking
Figure 4-30. NMR spectrum of RGD-PEO in d₆-DMSO. The downfield segment of the spectrum shows the acid proton at ~12.5 ppm, indicating that the N-terminal modification of RGD was successful. Also, there are now five triplet peaks instead of four in the amide proton region. The fifth triplet is Nₐ of the N-terminal glycine residue has changed from an amine to an amide. The ethylene glycol protons are a broad peak at 3.5 ppm.
In order to enable the coupling of the xHBPs to PVAm, the amino termini were modified with 3,6,9-trioxaundecanedioic acid to create an N-terminal acid. After cleavage, the xHBPs were not purified by reversed-phase HPLC since they were not soluble in any composition of acetonitrile and water. The xHBPs were purified by dissolving in DMSO and dialyzing for 24 hours against water using a regenerated cellulose membrane, MWCO 1000. The solution and precipitate within the membrane were then lyophilized. MALDI mass spectra of the xHBPs could not be acquired since a suitable solvent for the xHBPs could not be found that also allowed for co-crystallization with the matrix.

FT-IR and $^1$H-NMR were used to verify sample composition and purity of xHBP1 (figures 4-31 and 4-32) and xHBP2 (Figures 4-33 and 4-34). The FT-IR spectra of xHBP1-PEO and xHBP2-PEO shown in Figures 4-31 and 4-33, respectively, resembled their HBP1-PEO and HBP2-PEO counterparts. However, due to their increased alanine concentrations, there was noticeable increase in the relative absorptions for methyl groups—2975, 2879, and 1366 cm$^{-1}$. Compared to their HBP1 and HBP2 counterparts, respectively, xHBP1-PEO and xHBP2-PEO both exhibited shoulder peaks near 1735 cm$^{-1}$ due to the presence of a carboxylic acid and peaks at 1100 cm$^{-1}$ due to the presence of an ether group. See Tables 4-9 and 4-10, respectively. The NMR spectra of xHBP1-PEO and xHBP2-PEO are shown in Figures 4-32 and 4-34, respectively. The downfield segments of the spectra show the acid proton at 12.5 ppm, indicating that the
Figure 4-31. KBr pellet FT-IR spectrum for xHBP1-PEO.

Table 4-9. IR absorption assignments for xHBP1-PEO differing from HBP1.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800 - 3000</td>
<td>Increased relative absorption in ν(C-H) region</td>
</tr>
<tr>
<td>1729</td>
<td>Increased relative absorption in ν(C=O) region, sh</td>
</tr>
<tr>
<td>1366</td>
<td>Increased relative absorption for δₙ(C-H₃)</td>
</tr>
<tr>
<td>1250</td>
<td>ν(C-O), acid</td>
</tr>
<tr>
<td>1100</td>
<td>νₘ(C-O-C), ether</td>
</tr>
<tr>
<td>880</td>
<td>out-of-plane δ(O-H), acid; νₘ(C-O-C), ether</td>
</tr>
</tbody>
</table>

ν = stretching, νₛ = symmetric stretching, νₙ = asymmetric stretching, δ = deformation, ρ = rocking, sh = shoulder
Figure 4-32. NMR spectrum of xHBP1-PEO in d$_6$-DMSO. The downfield segment of the spectrum shows the acid proton at ~12.5 ppm, indicating that the N-terminal modification of xHBP1 was successful. The ethylene glycol protons are a broad peak at 3.5 ppm.
Figure 4-33. KBr pellet FT-IR spectrum for xHBP2-PEO.

Table 4-10. IR absorption assignments for xHBP2-PEO not attributed to HBP2.

<table>
<thead>
<tr>
<th>Wavenumber (cm⁻¹)</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2800 - 3000</td>
<td>Increased relative absorption in ν(C-H) region</td>
</tr>
<tr>
<td>1740</td>
<td>Increased relative absorption in ν(C=O) region, sh</td>
</tr>
<tr>
<td>1366</td>
<td>Increased relative absorption for δₙ(C-H₃)</td>
</tr>
<tr>
<td>1238</td>
<td>ν(C-O), acid</td>
</tr>
<tr>
<td>1100</td>
<td>νₚ(C-O-C), ether</td>
</tr>
<tr>
<td>964</td>
<td>out-of-plane δ(O-H), acid</td>
</tr>
</tbody>
</table>

ν = stretching, νₛ = symmetric stretching, νₚ = asymmetric stretching,
δ = deformation, ρ = rocking
Figure 4-34. NMR spectrum of xHBP2-PEO in d6-DMSO. The downfield segment of the spectrum shows the acid proton at ~12.5 ppm, indicating that the N-terminal modification of xHBP2 was successful. The ethylene glycol protons are a broad peak at 3.5 ppm.
N-terminal modification of the peptides were successful. The ethylene oxide protons appear as a broad peak at 3.5 ppm.

4.3.1.4. PVAm(PEP:Hex)

The PEO-peptides along with hydrophobic hexanoic acid ligands were incorporated into peptide surfactant polymers to produce PVAm(HBP1:Hex)[2:3], PVAm(xHBP1:Hex)[2:3], PVAm(HBP1:RGD:Hex)[1:1:3], PVAm(HBP2:Hex)[2:3], PVAm(xHBP2:Hex)[2:3], PVAm(HBP2:RGD:Hex)[1:1:3], and PVAm(RGD:Hex)[2:3]. These products were individually purified by dialysis (5000 MWCO), ensuring that each product contained only species with a molecular weight of greater than 5000 and no free PEO-peptides. The FT-IR spectrum of each surfactant polymer is shown in Figure 4-35.

Similar to the method used by Qiu et al. [4.32], a quantitative measure of the final molar peptide:hexanoyl ligand ratio was attempted using ¹H-NMR, shown in Figures 4-36 – 4-42. The peaks for the PVAm backbone were identified in Figure 4-8 and the peaks for the RGD, HBP1 and HBP2 peptides were identified in Figures 3-2, 4-12, and 4-7; therefore, the remaining unidentified peaks were assigned to the methyl and methylene groups in the hexanoyl chain. Particular attention was paid to the methyl peak at 0.8 ppm, which contains the hexanoyl methyl and, if present, V₁₁, V₁₂, I₁₂ and I₆ methyls. Of the polymers that did not contain aspartic acid, most did not have an acid proton in the vicinity of 12.5ppm, which shows that no unreacted PEP-PEO is present after dialysis. PVAm(xHBP2:Hex) was the only polymer that did not contain aspartate that displayed a
Figure 4-35. KBr pellet FT-IR spectra for the surfactant polymers. (A) PVAm(HBP1:Hex)[2:3], (B) PVAm(xHBP1:Hex)[2:3], (C) PVAm(HBP1:RGD:Hex)[1:1:3], (D) PVAm(HBP2:Hex)[2:3], (E) PVAm(xHBP2:Hex)[2:3], (F) PVAm(HBP2:RGD:Hex)[1:1:3], and (G) PVAm(RGD:Hex)[2:3].
Figure 4-36. 600 MHz $^1$H-NMR spectra of PVAm(HBP1:Hex)[2:3] in d$_6$-DMSO.
Figure 4-37. 600 MHz $^1$H-NMR spectra of PVAm(xHBP1:Hex)[2:3] in d$_6$-DMSO.
Figure 4-38. 600 MHz $^1$H-NMR spectra of PVAm(HBP1:RGD:Hex)[1:1:3] in $d_6$-DMSO.
Figure 4-39. 600 MHz $^1$H-NMR spectra of PVAm(HBP2:Hex)[2:3] in $d_6$-DMSO.
Figure 4-40. 600 MHz $^1$H-NMR spectra of PVAm(xHBP2:Hex)[2:3] in d$_6$-DMSO.
Figure 4-41. 600 MHz $^1$H-NMR spectra of PVAm(HBP2:RGD:Hex)[1:1:3] in d$_6$-DMSO.
Figure 4-42. 600 MHz $^1$H-NMR spectra of PVAm(RGD:Hex)[2:3] in $d_6$-DMSO.
very slight peak at 12.5ppm. Some xHBP2-PEO still remained after dialysis most likely due to its limited solubility.

4.3.2. Molecular Modeling of Peptide Surfactant Polymer

Molecular modeling software and the known crystal structure fragments of FN were used to find approximate size for HBP1, HBP2, and RGD, allowing an estimate of the maximum peptide density that could be achieved along the PVA backbone. The source of the active peptide sequence, WQPPRARI, of HBP1 is located in the 14th type III domain in FN. This domain, whose structure was solved by x-ray diffraction and downloaded from the protein data bank [4.38], is shown in Figure 4-43a with the active amino acid residues highlighted. Here the active sequence is relatively extended with the arginine residues extending outward and the tryptophan residue buried within the β sandwich structure. It is assumed that this β sandwich structure is largely responsible for the active sequence’s conformation since the active sequence’s ends are extended by being tethered between two β strands; therefore, HBP1 will not be as extended. However, if the arginines, which are probably responsible for the heparin binding function, remain extended outward, HBP1 should be active. Figure 4-43b shows the result of an energy minimization calculation performed in solution on HBP1 starting with the active sequence in the same conformation as in FN. The minimized conformation was not as extended, but as expected, the arginine residues extended out into solution. The distance between the alpha carbons of isoleucine and tryptophan was measured both in the FN crystal structure and the modeled HBP1 structure. The distance was 14.2Å for
Figure 4-43. Molecular modeling of HBP1. (a) Crystal structure of FN III$_{14}$ with the native active sequence of HBP1 highlighted. The active sequence is a random chain that links two β strands. (b) Conformation of energy minimized HBP1.
the former and 12.1Å for the latter, suggesting that HBP1 alone is not as extended as within the FN sequence. The final dimensions of HBP1 were measured to be 17Å×9Å×35Å (W×D×H). At this size, only one out of every three or four PVAm amino groups could accommodate HBP1 (based on a 3.5 Å amine spacing along the PVAm backbone).

For HBP2, the source of the active peptide sequence, SPPRRARVT is located in the 13th type III domain in FN. This domain, is shown in Figure 4-44a with the amino acid residues of the active sequence highlighted. The backbone of the active sequence is very extended as it is part of a β strand making up the β sandwich structure. The arginine residues extend outward into solution and the hydrophobic alanine and valine residues are buried within the β sandwich structure. It is assumed that the β sheet is largely responsible for the active sequence’s extended conformation since it is one of the strands composing the sheet. The synthesized peptide, HBP2, probably has a more random conformation since the β sandwich is not present to template the β strand conformation. However, if the arginines, which are probably responsible for the heparin binding function, remain extended outward, HBP2 should be active. Figure 4-44b shows the result of an energy minimization calculation performed in solution on HBP2 starting with the active sequence in the same conformation as in FN. The minimized conformation was not as extended, but as expected, the arginine residues extended out into solution. The distance between the alpha carbons of serine and threonine was measured both in the FN crystal structure and the modeled HBP2 structure. The distance was 23.8Å for the former and 20.3Å for the latter, suggesting that HBP2 is not as extended as the FN sequence. The final dimensions of HBP2 were measured to be 16Å×12Å×39Å
Figure 4-44. Molecular modeling of HBP2. (a) Crystal structure of FN III_{13} with the native active sequence of HBP2 highlighted. The active sequence is contained within one of the β strands. b) Conformation of energy minimized HBP2.
(W×D×H). At this size, only one out of every three or four PVAm amino groups could accommodate HBP2 (based on a 3.5 Å amine spacing along the PVAm backbone).

For RGD, the source of the active peptide sequence GRGDSPA is located in the 10th type III domain in FN. This domain, is shown in Figure 4-45a with the amino acid residues of the active sequence highlighted. The backbone of the active sequence is formed in a tight loop with the termini of the sequence held in place by two β strands in the β sandwich structure. The arginine-glycine-aspartate residues extend outward into solution. It is assumed that the constrained termini are responsible for the tight loop formed by the backbone of the active sequence. The synthesized peptide, RGD, probably has a more extended, random conformation since the termini of the linear nonapeptide are not constrained. However, if the arginine-glycine-aspartate residues, which are probably responsible for the integrin-binding function, remain accessible and extended outward, RGD should have some activity, as was shown in previous chapter (Section 3.4.1).

Figure 4-45b shows the result of an energy minimization calculation performed in solution on RGD starting with the active sequence in the same conformation as in FN. The minimized conformation was more extended, but as expected, the arginine-glycine-aspartate residues remain accessible to the solution. The distance between the alpha carbons of glycine and alanine was measured both in the FN crystal structure and the modeled RGD structure. The distance was 3.7Å for the former and 6.4Å for the latter, suggesting that RGD is not as extended as within the FN sequence. The final dimensions of RGD were measured to be 15Å×8Å×24Å (W×D×H). At this size, only one out of every three or four PVAm amino groups could accommodate RGD (based on a 3.5 Å amine spacing along the PVAm backbone).
Figure 4-45. Molecular modeling of RGD. (a) Crystal structure of FN III\textsubscript{10} with the native active sequence of RGD highlighted. The active sequence is contained within a tight loop constrained by two $\beta$ strands. (b) Conformation of energy minimized RGD.
The above dimensions of the peptides were used to predict the theoretical composition of a surfactant polymer and compared to the peptide ligand composition determined by $^1$H-NMR (Table 4-11). The measured ligand molar ratios were determined by the rationing the peptide and hexanoyl NMR integration of peaks. For measuring the ligand ratio of PVAm(HBP1:Hex) the I$_{γ1}$ peak (1.0 ppm, 1-$^1$H) was compared to the peak at 0.8 ppm, which contained 3-I$_{γ2}$ methyl protons, 3-I$_δ$ methyl protons, and 3 hexanoyl methyl protons. The ratio x/y was determined by $3 \cdot \int_{1.0\text{ppm}} - 6 \cdot \int_{1.0\text{ppm}}$, where $\int$ is the symbol used for NMR integration, and x and y are defined in Figure 4-36.

For PVAm(xHBP1:Hex) the A$_β$ peak (1.2 ppm, 9-$^1$H), which contained the methyls of three different alanines, was compared to the peak at 0.8 ppm, which contained 3-I$_{γ2}$ methyl protons, 3-I$_δ$ methyl protons, and 3 hexanoyl methyl protons. The ratio x/y was determined by $\int_{1.2\text{ppm}} / (3 \cdot \int_{0.8\text{ppm}} - 2 \cdot \int_{1.2\text{ppm}})$.

For PVAm(HBP1:RGD:Hex) the I$_{γ1}$ peak (1.0 ppm, 1-$^1$H) was compared to the peak at 0.8 ppm, which contained 3-I$_{γ2}$ methyl protons, 3-I$_δ$ methyl protons, and 3 hexanoyl methyl protons and also compared to the A$_β$ peak (1.2 ppm), which contained the one methyl from RGD and one from HBP1. The ratio x/y was determined by $3 \cdot \int_{1.0\text{ppm}} / (\int_{0.8\text{ppm}} - 3 \cdot \int_{1.0\text{ppm}})$, and x/w was determined by $3 \cdot \int_{1.0\text{ppm}} / (\int_{1.2\text{ppm}} - 3 \cdot \int_{1.0\text{ppm}})$, where w, x, and y are defined in Figure 4-38.

For PVAm(HBP2:Hex) the T$_γ$ methyl peak (0.95 ppm, 3-$^1$H) was compared to the peak at 0.8 ppm, which contained 3-V$_δ1$ methyl protons, 3-V$_δ2$ methyl protons, and 3 hexanoyl methyl protons. The ratio x/y was determined by $\int_{0.95\text{ppm}} / (\int_{0.8\text{ppm}} - 2 \cdot \int_{0.95\text{ppm}})$. 

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Table 4-11. Surfactant polymer ligand ratios calculated from $^1$H-NMR.

<table>
<thead>
<tr>
<th>Material</th>
<th>Measured Ratio</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x, HBP</td>
<td>w, RGD</td>
<td>y, Hex</td>
</tr>
<tr>
<td>PVAm(HBP1:Hex)</td>
<td>1</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>PVAm(xHBP1:Hex)</td>
<td>1</td>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>PVAm(HBP1:RGD:Hex)</td>
<td>1</td>
<td>1.1</td>
<td>0.5</td>
</tr>
<tr>
<td>PVAm(HBP2:Hex)</td>
<td>1</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>PVAm(xHBP2:Hex)</td>
<td>1</td>
<td>0</td>
<td>0.4</td>
</tr>
<tr>
<td>PVAm(HBP2:RGD:Hex)</td>
<td>1</td>
<td>1.7</td>
<td>3.8</td>
</tr>
<tr>
<td>PVAm(RGD:Hex)</td>
<td>0</td>
<td>1</td>
<td>0.6</td>
</tr>
<tr>
<td>PEP:Hex feed ratio</td>
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<td>3</td>
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</tr>
<tr>
<td>Theor. modeling max (mol %) x+w, PEP</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Theor. (mol %) y, Hex</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Theor. (mol %) z, amino groups</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
For PVAm(xHBP2:Hex) the T\textsubscript{γ} methyl peak (0.95ppm, 3-\textsuperscript{1}H) was compared to the peak at 0.8 ppm, which contained 3-V\textsubscript{δ1} methyl protons, 3-V\textsubscript{δ2} methyl protons, and 3 hexanoyl methyl protons. The ratio x/y was determined by \( \int_{0.95\text{ppm}} \div (\int_{0.8\text{ppm}} - 2 \times \int_{0.95\text{ppm}}) \).

For PVAm(HBP2:RGD:Hex) the T\textsubscript{γ} methyl peak (1.0ppm, 3-\textsuperscript{1}H) was compared to the peak at 0.8 ppm, which contained 3-V\textsubscript{δ1} methyl protons, 3-V\textsubscript{δ2} methyl protons, and 3 hexanoyl methyl protons and also compared to the A\textsubscript{β} peak (1.2ppm), which contained the one methyl from RGD and one from HBP2. The ratio x/y was determined by \( \int_{1.0\text{ppm}} \div (\int_{0.8\text{ppm}} - 2 \times \int_{1.0\text{ppm}}) \), and x/w was determined by \( \int_{1.0\text{ppm}} \div (\int_{1.2\text{ppm}} - \int_{1.0\text{ppm}}) \).

For PVAm(RGD:Hex) the P\textsubscript{γ} peak (2.1ppm, 2-\textsuperscript{1}H) was compared to the peak at 0.8 ppm, which contained 3 hexanoyl methyl protons. The ratio x/y was determined by \( 3 \times \int_{2.1\text{ppm}} \div (2 \times \int_{0.8\text{ppm}}) \). It should be noted here that there is significant error in NMR peak integrations, and this error is compounded through the determination of x/z and x/w.

**4.3.3. Modified Surfaces**

**4.3.3.1. Contact Angle**

Water contact goniometry was used to show that the surfactant polymers were surface active on OTS-modified glass. The OTS self-assembled monolayer was created on glass coverslips to be used as a model hydrophobic substrate for the testing of polymer surfactants. This monolayer modified the water contact angle of the surface to 102 ± 2° from about 20° for an unmodified glass surface (Table 4-12). After 24-hour surfactant polymer absorption onto OTS-modified glass, the surfactant-modified surfaces had an
Table 4-12. Contact angle measurements for surfactant polymer adsorbed surfaces.

<table>
<thead>
<tr>
<th>Material</th>
<th>Avg. advancing angle</th>
<th>Avg. min. receding angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTS</td>
<td>102 ± 2°</td>
<td>98 ± 2°</td>
</tr>
<tr>
<td>PVAm(HBP1:Hex)</td>
<td>71 ± 26°</td>
<td>40 ± 20°</td>
</tr>
<tr>
<td>PVAm(xHBP1:Hex)</td>
<td>17 ± 5°</td>
<td>4 ± 2°</td>
</tr>
<tr>
<td>PVAm(HBP1:RGD:Hex)</td>
<td>67 ± 19°</td>
<td>35 ± 21°</td>
</tr>
<tr>
<td>PVAm(HBP2:Hex)</td>
<td>71 ± 26°</td>
<td>21 ± 13°</td>
</tr>
<tr>
<td>PVAm(xHBP2:Hex)</td>
<td>49 ± 12°</td>
<td>6 ± 3°</td>
</tr>
<tr>
<td>PVAm(HBP2:RGD:Hex)</td>
<td>52 ± 23°</td>
<td>14 ± 10°</td>
</tr>
<tr>
<td>PVAm(RGD:Hex)</td>
<td>68 ± 24°</td>
<td>34 ± 22°</td>
</tr>
</tbody>
</table>
average advancing water contact angle significantly lower than uncoated OTS. The average minimum receding angles were to evaluate the contact angle hysteresis of the surface coatings. All of the surfaces, except for OTS, had markedly lower receding contact angles compared to their corresponding advancing angles.

4.3.3.2. Endothelialization

HPAEC were shown to attach and proliferate on the surfactant-modified surfaces in a manner similar to fibronectin-coated glass controls. Three hours after attachment, more cells were observed attached to the materials containing heparin binding peptides when compared to the FN-coated glass. Laser scanning confocal microscopy at three hours (Figures 4-46A, D, G, J, and M) also showed more focal adhesions and long, well-organized actin stress fibers for cells on PVAm(HBP1:RGD:Hex) or PVAm(HBP2:RGD:Hex) when compared to FN-coated glass. After 6 hours (Figures 4-46B, E, H, K, and N), all surfaces were comparable showing good cell density, good actin fiber organization, and many focal adhesions. After 2 days, the cells on PVAm(HBP1:Hex) or PVAm(HBP2:Hex) were much fewer in number compared to the FN and RGD containing materials and did not show many focal adhesions nor good actin fiber organization. At 2 days, the FN surface appears to be closer to a state of confluence compared to the PVAm(HBP1:RGD:Hex) or PVAm(HBP2:RGD:Hex).
Figure 4-46. Focal adhesion and stress fiber formation of EC on FN (A-C), PVAm(HBP1:Hex) (D-F), PVAm(HBP2:Hex) (G-I), PVAm(HBP1:RGD:Hex) (J-L), and PVAm(HBP2:RGD:Hex) (M-O) at 3 hr (A, D, G, J, M), 6 hr (B, E, H, K, N), and 48 hr (C, F, I, L, O). HPAEC are stained for actin stress fibers (red), and the focal adhesion protein vinculin (green). Images obtained using scanning confocal microscopy (60x objective).
4.4. Discussion

Proposed here is a biomaterial surface modification for the facilitation of endothelial cell attachment and proliferation. The surfactant polymer allows for this modification to occur as a simple, one-step dip-coating process. Modifications by the adsorption of amphiphilic molecules onto hydrophobic substrates have been shown previously to be stable even under physiological competitive adsorption and shear conditions [4.45-52]. The designed surfactant polymer consists of a PVAm backbone whose amine functional groups allow for the selective attachment of pendant hydrophobic groups for surface activity and pendant hydrophilic, biologically active peptides. The use of the same functional group for attaching different pendant groups allows for the modulation of the hydrophobe to hydrophile ratio in order to optimize surface activity [4.32, 46]. The chain length of the hydrophobe can also be varied for surface activity optimization and the chemical composition of the hydrophobe can be tailored to the target material surface to increase the thermodynamic force for adsorption, as in using a fluorocarbon chain for adsorption onto polytetrafluoroethylene [4.53]. The hydrophile is selected for the desired surface properties including, but not limited to, steric repulsion of protein adsorption [4.48] or EC integrin binding [4.33]. This chapter introduces a new biologically functional hydrophilic pendant group—HBPs for binding with EC HSPG.

The surfactant polymer begins with the synthesis of a well-defined and pure polymer backbone. PVAm was synthesized according to Qiu et al. [4.32]; however instead of synthesizing the monomer, it was procured from Aldrich. The free radical
The polymerization of N-vinyl formamide proceeded until the polymer precipitated. PNVF was characterized by $^1$H-NMR (Figure 4-3) and FTIR (Figure 4-2) in order to track synthetic modifications. GPC (Figure 4-4) was used to determine the molecular weight of PNVF to be $M_n = 16,000 \pm 6,000$ g/mol with a polydispersity of $1.5 \pm 0.8$, which will correspond to PVAm with a molecular weight of $M_n = 9,600$ g/mol. The narrow weight distribution achieved may be due to the precipitation of PNVF during polymerization, thus limiting polymerization to a certain point. PNVF was then subjected to basic hydrolysis.

Complete conversion of PNVF to PVAm•HCl is displayed in the FTIR spectrum (Figure 4-5) by the loss of the amide I absorbance at 1661 cm$^{-1}$ and at the appearance of protonated amine peaks at 2004 cm$^{-1}$. Complete hydrolysis is also illustrated in the $^1$H-NMR spectrum (Figure 4-6) by the lack of a formamide proton at 8.0 ppm. Ion exchange chromatography was used to convert PVAm•HCl to PVAm. Complete conversion is illustrated by the lack of protonated amine peaks in the FTIR spectrum (Figure 4-7) and by the upfield shifts of the methylene and methine protons in the $^1$H-NMR spectrum (Figure 4-8).

The occurrence of heparin-binding sequences is quite common in nature. The increased content of basic amino acid residues, most importantly arginine, is shown to be crucial in binding to heparin, a negatively charged glycosaminoglycan [4.23]. Also important is the peptide sequence and the amount of amino acid spaces (denoted as X) between these basic residues (denoted as B) in such consensus sequences as XBBBXXBX and XBBXBX [4.22, 54, 55]. Peptide sequences of this variety bind to heparin in the micromolar range [4.23]. A variety of heparin-binding sequences exist such as those in antithrombin III [4.56, 57] and some snake venoms [4.58], but these
sequences are not a mimic of EC HSPG interaction with the ECM. Other ECM proteins like laminin have heparin-binding sequences [4.59], but fibronectin, being the quintessential EC adhesive ECM protein, has the HBP sequences most logically chosen to mimic. Mooradian et al. identified two peptide sequences from the HepII heparin binding region of fibronectin that support epithelial cell adhesion: 1) WQPPRARI and 2) SPPRRARVT [4.31]. Mohri et al. showed that the first peptide inhibited the 29kDa heparin binding fragment of fibronectin to heparin-sepharose with an $IC_{50} = 210 \pm 37 \mu M$ [4.60]. Ingham et al. showed a peptide containing the second sequence bound to heparin with a $K_d$ of 41 $\mu M$ [4.61], and the extended native 22-residue peptide sequence bound with a $K_d$ of 0.3 $\mu M$ [4.30]. The three arginines in the second sequence have been shown to be crucial for FN-III$_{13}$ binding to heparin as mutations at these locations obliterated binding in 0.15 M NaCl [4.62].

Our biomimetic surfactant polymers have HBP that include the functional heparin-binding sequences described above: 1) WQPPRARI and 2) SPPRRARVT [4.31]; however, in order to minimize any electrostatic repulsion between HSPG and negatively charged C-terminal carboxylates, HBP1 and HBP2, through the use of the Knorr solid phase support resin, were synthesized to have neutral, amidated C-termini. The design of HBp1 and HBP2 includes a short pentapeptide added to the N-terminus, GSSSG in the case of HBP1 and GSWSG in the case of HBP2. (Tryptophan is sometimes added as UV absorber/fluorescent marker for diagnostic purposes. (Data not shown.)) The pentapeptide, with its hydrophilic serine residues, discourages interactions with the hydrophobic hexanoyl legs, the surfactant polymer backbone, and the biomaterial surface, elevating the active sequence above the biomaterial surface and
preserving function [4.33]. Introducing hydrophilic PEO spacers into HBP1 and HBP2 for linking the peptide to the backbone also further elevates the functional sequence above the surface. A hydrophilic PEO spacer was used here instead the five methylene unit spacer created by glutaric dialdehyde used previously [4.33]. The surfactant polymers synthesized by Qiu et al. showed that an aliphatic chain five carbons long was sufficient to promote surface activity [4.32]. A five methylene unit spacer could adsorb onto the biomaterial surface and interfere with the desired parallel adsorption of the surfactant polymer chains, disallowing the desired interdigitation of the hexanoyl legs.

To confirm the well-defined synthesis of the HBP1, HBP2, and RGD peptides, they were synthesized on a solid support resin, cleaved and deprotected under acidic conditions, and purified using rp-HPLC (Figures 4-9 and 4-14). The pure peptide fractions were isolated using optimized gradient conditions and collected for further characterization to confirm structure and purity. NMR (Figures 4-12, 4-13, 4-17, and 3-2) and IR (Figures 4-11, 4-16) confirmed the structure of the peptides, and mass spectroscopy (Figures 4-10, 4-15, and 4-18) confirmed that only the desired peptides were synthesized and purified. In the case of the RGD peptide, a small amount of peptide still protected by Pbf was still present. This was not due to ineffective synthesis of the peptide but due to a less than 100% effective deprotection. In the future steps of synthesizing PEO-peptides, the Pbf-containing components can be minimized by using fresh cleavage reagents and scavengers and by extending the time of the deprotection.

Most often, PEO conjugation is done in solution in methods analogous to those in Boger et al. [4.63]; however in this case, coupling of the PEO spacer was done in the solid phase. This method takes advantage of the PEO-peptide conjugate product still
being tethered to the solid support. Total removal of any remaining PEO diacid, which if not removed would act as a crosslinker of the PVAm backbone, is easily accomplished through multiple washings and filtrations of the resin. Also, doing the conjugation in the solid-phase takes advantage of pseudodilution [4.64]. Pseudodilution is a kinetic phenomenon, where inter-peptide reactions are not favored while coupled to the solid support, since for two solid-supported peptides to interact, the polymer backbones of the crosslinked solid support resin must raptate in order to bring the two peptides within proximity for reaction. The occurrence was measured for an average resin to be between $2.6 \times 10^{-3}$ and $1.1 \times 10^{-3}$ s$^{-1}$ [4.65], which is relatively slow when compared to solution phase reactions. The principal of pseudodilution is most effectively applied in the case of forming cyclic molecules because the frequency of intramolecular interaction is greater than that of intermolecular interaction, and once the cycle is formed, the molecule is no longer free to react with other molecules [4.66-69]. Despite pseudodilution, it is still possible for two molecules in the solid phase to react to form dimers [4.65, 70, 71]. Ashraf et al. set out to form dimers, and did so with a Wang resin of average substitution with yields ~50%, by using long reaction times and a peptide- homobifunctional linker ratio of 2:1 [4.70]. To avoid dimer formation Nguyen-Trung et al. used a solid support with an extremely low substitution ratio (0.28 μmol/g), an impractically low ratio when concerned with making peptides in high yields for biomaterial modification [4.72]. Crowley et al. found the amount of intraresin reactions to be directly proportional to the degree of resin substitution [4.73].

The approach in this chapter to react one end of a homobifunctional linker, to a peptide on the solid support, was not only to take advantage of pseudodilution, but also to
use the PEO linker : activator : peptide ratio of 5 : 1 : 0.25 to minimize dimer formation. Since the linker is homobifunctional, there are actually 10 equivalents of carboxylates in solution, and the frequency of activated carboxylates is 0.1. In solution, the amounts of unactivated, mono-activated, and di-activated PEO linkers are 4.05, 0.45, and 0.05 equiv., and 0.45 equiv. of mono-activated PEO is more than enough to react with 0.25 equiv. of peptide. Having a large excess of unactivated carboxylates could promote anhydride formation, but this is not a concern since anhydrides can readily react with the N-terminus to give the desired product [4.74]. The occurrence of reaction of mono-activated PEO is 90%, when compared to di-activated PEO. The additional effect of pseudodilution should further reduce the amount of dimer to well below 10%. Evidence of successful PEO-peptide conjugation was observed using IR spectroscopy (Figures 4-21, 25, 29, 31, and 33) and NMR spectroscopy (Figures 4-22, 26, 30, 32, and 34). MALDI mass spectroscopy (Figures 4-20, 24, and 28) confirmed the successful conjugation, the low incidence of dimers, and the complete absence unreacted peptide.

Molecular modeling was used to estimate the size of the peptides in order to determine the amount of peptide substitution that could be achieved along the PVAm backbone. The size determined for the HBP1, HBP2, and RGD peptides was such that only one out every four amines along the backbone could accommodate a peptide; however, since the conjugation is random along the backbone, then the peptide substitution along the backbone should be less than 25%. If a peptide was five, six, or seven amines down the backbone from another peptide, a third peptide would be sterically hindered from conjugating with the backbone at any location between the first two peptides. This explains the lower substitution achieved previously despite the large
excess of peptide used [4.75]. For these peptides, the highest density along the backbone for this randomly substituted polymer is most likely an average of \(1/4, 1/5, 1/6,\) and \(1/7,\) or 18\% (displayed in Table 4-11). Since surfactant polymer synthesis was performed in two steps by first conjugating the peptides to the backbone, followed by coupling of hexanoic acid, it is possible that the peptides filled the space along the backbone and sterically hindered the coupling of hexanoic acid, which would explain the lower than expected hexanoyl composition reported in Table 4-11.

Determination of the \(w:x:y:z\) ratio (coefficient are defined in Figures 4-36 - 4-42) of the peptide surfactant polymers proved difficult using \(^1H\)-NMR peak integration because the peptide protons obscured the peaks of the PVAm backbone, preventing the determination of “z,” and because of the large amount of error associated with NMR peak integration, error that is further compounded by the calculations performed in Section 4.3.2. An alternative could involve NMR analysis after each step of the surfactant polymer synthesis [4.76]. NMR peak integration after the first step could give the mol \% composition of peptide in the polymer by comparing to backbone protons. NMR peak integration after the second step would then give the \(x:y\) ratio; however, peptide peaks commonly overlap with PVAm peaks preventing analysis after the first step. Wang used the elemental composition determined from XPS (elemental analysis could also be used) to determine the \(x:y:z\) ratio [4.46, 53]; however, the elemental percentages derived from these experiments also have a large amount of error translating to a large error in \(x:y:z\). Since the HBP1, HBP2, and RGD peptides contain tryptophan, UV spectroscopy could be used to determine the weight percentage of \(x\) in the surfactant polymer. Coupled with the \(x:y\) ratio determined from NMR, \(z\) can be derived.
With the exception of PVAm(xHBP2:Hex), after dialysis, $^1$H-NMR spectra of the surfactant polymers showed the presence of peptide peaks and hexanoyl methyls, yet no carboxylic acid peak at ~12.5ppm, indicating successful conjugation to the PVAm backbone (Figures 4-36 - 4-42). A well-defined surfactant polymer was synthesized because of the selectivity of the ligands to react only with the amines of the PVAm backbone. Only the acid of hexanoic acid and the PEO-acid at the peptide N-terminus could react with the amines. The exception is RGD-PEO, which contains two carboxylates. For this peptide, aspartic acid could also be coupled to the PVAm backbone, making the biologically active RGD tripeptide sequence inaccessible. This may explain why the PVAm(RGD:Hex) prepared in the manner described here did not support cell growth as well as FN [4.77], while a PVAm(RGD:Hex) prepared in a more selective manner did rival that of FN [4.78]. Also the two acids in RGD-PEO could act as a PVAm crosslinker, an undesirable result. The fact that RGD-PEO has an additional acid could explain why NMR analysis showed a higher RGD peptide concentration compared to HBP in the case of the PVAm(HBP1:RGD:Hex) and PVAm(HBP2:RGD:Hex) (Table 4-11). Peptide synthesis of the RGD peptide could be performed with Fmoc-Asp(OBzl)-OH instead of Fmoc-Asp(OtBu)-OH. Unlike the tert-butyl ester, the benzyl ester is not removed under the deprotection conditions of 85% TFA; it requires hydrofluoric acid or trifluoromethane sulfonic acid (TFMSA). The aspartate would remain protected as RGD(OBzl)-PEO, and would not be coupled to PVAm. Once the RGD surfactant polymer is synthesized, the benzyl ester can be removed with TFMSA.
Contact angle goniometry showed through the large decrease in the surface contact angle for the surfactant modified surfaces that the surfactant polymers were able to adsorb onto the surface of OTS, but there is still room for improvement. Given the design of the surfactant polymers, the surfactant polymer adsorption can be tuned though the hydrophobe:hydrophilic ratio [4.46], the length of the hydrophobe [4.53], and/or the density of the hydrophile. By increasing the amount of the hydrophobe along the backbone, surface activity can be increased [4.46]; however, if the hydrophobe content is increased too much, then there will be no space between the hydrophobic legs to allow interdigitation with a neighboring surfactant polymer’s legs [4.79]. Interdigitation is an important factor in the strength of the polymer’s adsorption because then the polymer surfactant’s adsorption is not only stabilized through its interaction with the material surface but also through interactions with neighboring adsorbed polymers.

As shown in Figure 4-47, up to a certain point the length of the hydrophobic chain can also be increased to improve surface activity. The length should be increased to maximize surface activity but not too much to disrupt the packing of the hydrophile as depicted in Figure 4-47B. In Figure 4-47A the hydrophobic chain is too short for interdigitation, and in Figure 4-47C the hydrophobe is so long that the canopy of the hydrophile no longer covers the entire surface. Also if the degree of substitution of the hydrophobe is too great as in Figure 4-47D, interdigitation is prevented, and the canopy is also disrupted. The currently used hexanoyl chains have a length of ~6Å, and the peptides used in this study have a major radius of ~8Å and a minor radius of ~5Å. Figure 4-47A most likely describes the surfactant polymers that were synthesized. Future
Figure 4-47. Models of the top view of surfactant polymers adsorbed on the surface. The horizontal lines represent the PVAm backbone. The vertical lines represent the length of the alkyl legs (l), and the gray circles represent the hydrophilic peptide. (A) depicts when the alkyl chain length is too short to interact with the alkyl chains of a neighboring surfactant polymer. (B) depicts alkyl leg interdigitation. (C) depicts alkyl leg interdigitation while the alkyl chain length is greater than the surface projection of the peptide. (D) depicts when interdigitation is not possible due to either increased alkyl composition (left side) or incompatible spacing (right side).
surfactant polymers may have improved surface activity if the alkyl chains are increased to octanoyl, or even undecanoyl chains.

If the increasing the hydrophobe length to the previously recommended level is impractical due to a very large hydrophile, then the hydrophile density along the PVAm backbone can be decreased without disrupting the canopy as shown in Figure 4-48B. Figure 4-48A shows the model of surface packing of the current polymers. Using the design in Figure 4-48B, hydrophile density along the backbone is 58% that of Figure 4-48A, while Figure 4-48A has 58% of the hydrophobic legs per unit area that are is Figure 4-48B. Both regimes, however, have the same unit lengths of hydrophobe per unit area, which can also be thought of as the methylene surface density. Other regimes can be created with identical peptide and methylene surface densities \textit{ad infinitum} (Figure 4-48C). If hexanoyl (length \sim 6Å) is the preferred hydrophobe for the surfactant polymers using HBP1, HBP2, and/or RGD (major radius \sim 8Å, minor radius\sim 5Å), then using one of the regimes depicted by the second or third lines in Figure 4-48C is preferred. These two regimes correspond 58% and 38% peptide loading along the PVAm backbone, respectively.

The EC growth and proliferation studies showed that the surfactant-modified surfaces supported cell attachment and growth over a 48-hour period. After 3 hours, phase contrast microscopy showed that surfactant-modified surfaces containing HBP allowed for more EC adherence, when compared to the FN control surface, and more focal adhesions were seen by laser scanning confocal microscopy. It could then be concluded that these materials are well suited for EC attachment. However after growing to a sufficient cell density that approaches confluency, ECs on FN and RGD-containing
Figure 4-48. Models of the top view of surfactant polymers adsorbed on the surface. The horizontal lines represent the PVAm backbone. The vertical lines represent the alkyl legs, and the gray circles represent the hydrophilic peptide. (A) depicts when the hydrophile is at maximum density along the backbone. (B) depicts when the hydrophile density along the backbone is ~58%. Both configurations have identical hydrophile surface density and identical methylene surface density. All lines in (C) represent the backbone, and configurations with progressively lower backbone hydrophile loading with identical hydrophile and methylene surface densities can be created *ad infinitum*. Black dots indicate attachment between a peptide and a backbone.
surfaces begin to form cell-cell contacts and assume the cobblestone morphology, but the ECs on the surfaces with HBPs only did not approach confluence. This suggests that ECs also require surface interaction via integrins for complete attachment. It has been suggested that surface attachment happens in a complementary way through both the heparan sulfate proteoglycans and integrins [4.29, 80-87].

As more is being discovered regarding cell surface HSPG, also called syndecans, it is becoming known that they are very important to the function of the cell. Recently it has become known that syndecans are involved in cell signaling [4.88-93]. Cytosolic domains of syndecans interact with integrins and tyrosine kinase receptors [4.94]. Tkachenko et al. found that Syndecan-4 can cluster on EC surface, initiating a signaling cascade and inducing migration [4.95]. Syndecans also have an important role in the strength of cell anchorage [4.96]. Syndecans regulate microdomains of receptors [4.89]. The extracellular protein of syndecan-1 complexes with and regulates αvβ5 integrin [4.97]. Syndecan-4 acts cooperatively to form focal adhesions, interacting with the actin cytoskeleton in a way complementary to integrins [4.98]. It has been discovered that the cytoplasmic domain of syndecan-2 has a binding domain for Ezrin, a structural protein that crosslinks plasma membrane proteins and the actin cytoskeleton [4.99].

Peterson et al. has called into doubt that syndecans bind with FN’s Hep II domain, the domain that contains both HBP1 and HBP2, by showing that only blocking antibodies for α4β1 were able prevent cell attachment to surfaces prepared with FN Hep II [4.100]. This would explain the result by Sagnella et al. that soluble heparin was not able to prevent cell attachment to the PVAm(HBP1:Hex) surface; however, it does not explain why heparin inhibited cell attachment to PVAm(HBP2:Hex) [4.77]. It may be possible
that the surfaces prepared by Peterson et al. only exposed the α4β1 binding sites of FN’s Hep II, while obscuring the heparin binding sites. The crystal structure of Hep II shows that the α4β1 and the heparin binding sites are on opposite faces of the molecule [4.38].

It was hypothesized that a material whose surface expresses an affinity for heparin will bind the heparan sulfate proteoglycans within the EC glycocalyx and promote endothelialization, leading to a nontthrombogenic surface. A model surface-modification involved the heparin-binding regions of the ECM protein, FN. Two such peptide sequences from these regions are WQPPRARI (HBP1) and SPPRRARVT (HBP2). ECM mimicking surfactant polymers containing the biologically active peptides were prepared. The synthesis of the surfactant polymers, their surface active properties, and their ability to support ECs were characterized. The successful growth and attachment of ECs demonstrates the potential of these surfactant polymers. The surfactant polymer can be used to study specific cellular interactions with surfaces and could possibly be used to produce nontthrombogenic, and hence, more enduring cardiovascular biomaterials. In conclusion, surfaces modified with HBP can be used to promote EC attachment and growth via interactions with the HSPG, and hence may be utilized in the production of a hemocompatible surface.

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4.6. References


Saphenous-Vein Disease - an Unrecognized Cause of Vein Bypass Failure.


4.44. *Unpublished result*, A MALDI-TOF mass spectrum was acquired for a newly cleaved peptide, showing equal intensity for the two species: 

\[ [M + H]^+ \] and \[ [M + Pbf + H]^+ \]. However, separation of the two fractions and integration of their peaks at both 220 nm and 280 nm using analytical scale rp-HPLC showed that the actual relative concentration was closer to 90:10. Therefore, MALDI-TOF over exaggerates species with Pbf groups.


4.75. Anderson, E.H., *Biomimetic polymers with peptide ligands designed for endothelial cell growth on cardiovascular implants*, in Department of Macromolecular Science. 2002, Case Western Reserve University: Cleveland. p. 120.


Chapter 5. DESIGN, SYNTHESIS, AND CHARACTERIZATION OF A LIGAND THAT INCORPORATES BOTH THE SYNERGY AND INTEGRIN-BINDING PEPTIDES

5.1. Introduction

In 2003 coronary heart disease killed or contributed to the death of 653,000 individuals [5.1]. It is and has been the leading cause of death for over 75 years. Currently, the preferred small-diameter vessel replacements are still autogenous vessels [5.2]; however, in many patients, they are of poor quality or nonexistent due to previous procedures [5.3]. There are also complications with using veins, which have a different anatomy than that of arteries, to perform an arterial physiological role under increased flow and pressure conditions [5.4]. There is clearly a need for successful, long-term, synthetic, small-diameter, vascular grafts.

Many materials have been evaluated as grafts including, but not limited to, Dacron (poly(ethylene terephthalate), PET), GoreTex (expanded poly(tetrafluoroethylene), ePTFE), polyethylene (PE), nylon, polydimethylsiloxane (PDMS), and polyurethane [5.5]. Numerous attempts have been made to modify the previously mentioned materials in an effort to improve their interfacial properties with blood. The rationale is to use a material that already possesses desirable bulk properties such as strength, durability, and compliance and then modify its surface to reduce its thrombogenicity [5.6]. Perhaps the most promising surface modification is to support a
layer of vascular endothelium on the interior of the graft. The endothelium, composed of endothelial cells (EC), is the natural lining of blood vessels, and therefore has an inherent resistance to thrombosis [5.7-13]. However, human EC do not adhere to the hydrophobic surfaces of polymer grafts [5.14-18]. An interface must be created that both adheres to the polymer surface and supports EC attachment, growth, and proliferation. These interfaces usually incorporate the cell adhesive peptide, arginine-glycine-aspartic acid (RGD), which is a native peptide sequence found in many extracellular matrix (ECM) proteins, including the III10 domain of fibronectin (FN) where it binds to the EC’s $\alpha_5\beta_1$ integrin. One problem is that RGD cannot completely mimic FN’s ability to bind to the $\alpha_5\beta_1$ integrin [5.19-21].

It has been shown that FN domain III9’s presence alongside domain III10 imparts a 2 order of magnitude increase in affinity for the $\alpha_5\beta_1$ integrin [5.22-24]. In particular, the PHSRN synergy peptide sequence from FN III9 has been shown to significantly contribute to the $\alpha_5\beta_1$ integrin’s affinity for FN [5.22, 25]. X-ray crystallography showed that the PHSRN sequence is on the same side of the FN molecule as FNIII10’s RGD sequence, and the distance between the two sequences is 30 – 40 Å [5.26]. By inserting extra amino acids between FNIII9 and FNIII10, Grant et al. showed that integrin affinity is dependent on the distance between and the orientation of the two peptide sequences and also showed increasing this distance has a negative effect on cell attachment, cell spreading and focal adhesion kinase phosphorylation [5.27]. Therefore, incorporation of the PHSRN peptide into our biomimetic design should enhance affinity to EC integrins given that it is spaced the proper distance from the RGD-containing peptide.
A design of a vascular graft surface modification that will promote endothelialization is proposed. An amphiphilic polymer, which will modify a graft through a simple dip-coating process, will be synthesized (Figure 5-1). This polymer design consists of a poly(vinyl amine) (PVAm) backbone with hydrophobic alkyl branches for adsorption onto the material surface. The hydrophilic groups consist of an RGD containing peptide that is attached to the PHSRN synergy peptide sequence using a poly(ethylene oxide) chain (PEO). This PEO molecule that tethers together the two peptides is of a certain size, whose molecular weight is chosen to provide the same end-to-end distance as the distance of separation between the two cell adhesive peptide sequences in the native protein, FN. Incorporation of these two cell adhesive peptides with the proper separation should result in increased cell receptor affinity and an increased cell response. In this study, the hydrophile of the two cell adhesive peptides connected by a PEO tether is synthesized and characterized using mass spectrometry, and nuclear magnetic resonance spectroscopy.

5.2. Materials and Methods

5.2.1. Materials

For solid phase peptide synthesis (SPPS) all protected and unprotected amino acids (Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Pro-OH, and Fmoc-Ser(tBu)-OH) were
Figure 5-1. Design of a biomimetic surfactant polymer that incorporates synergistic binding to EC integrins. Attached to a PVAm backbone (A) are hydrophobic legs (B) for adsorption onto the material surface and integrin-binding, RGD-containing peptides (C, colored in red). The integrin-binding, PHSRN-containing peptides (D, colored in purple) are tethered to the RGD-containing peptides with a poly(ethylene oxide) chain (E, colored in gold) that provides a 4.4 nm separation (from arginine $C_\alpha$ to arginine $C_\alpha$) of the two active peptide sequences.
purchased from Anaspec, Inc. (San Jose, CA). The N, N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), dichloromethane (DCM), and N, N-diisopropylethylamine (DIPEA) solvents were SPPS grade and purchased from Applied Biosystems (Foster City, CA). The Knorr-polystyrene solid support resin (Fmoc-Knorr-PS), the activating agent cocktail, HBTU ((2-(1H-benzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate) with HOBt/DMF (0.5 M 1-hydroxybenzotriazole in N, N-dimethylformamide), and the cleavage/deprotection reagent, trifluoroacetic acid (TFA), were also purchased from Applied Biosystems. The scavengers for the peptide cleavage reaction (phenol, 1,2-ethanedithiol (EDT), and triisopropylsilane (TIS)) along with piperidine were purchased from Aldrich. All SPPS and peptide cleavage solvents and reagents were used as received. For peptide N-terminal modification, the poly(ethylene glycol) bis(carboxymethyl) ether (3,6,9-trioxaundecanedioic acid, Mn = 222 g/mol), poly(ethylene oxide) (Mn ~ 600 g/mol, PEO600), and NHS (N-hydroxysuccinimide) were used as obtained from Aldrich. The EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide) was purchased from Sigma Chemical Co. (St. Louis, MO). Spectra Pro 3 regenerated cellulose dialysis membranes with 500 and 3000 molecular weight cutoffs (MWCO) were obtained from Fisher.

For FT-IR spectroscopy, potassium bromide (KBr) was IR grade and acquired from Fisher. In the nuclear magnetic resonance spectroscopy experiments deuterated water (D$_2$O) and dimethyl-d$_6$ sulfoxide (DMSO-d$_6$) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Also, α-cyano-4-hydroxycinnamic acid was obtained from Aldrich as the MALDI-MS matrix.
Ultra pure water was produced by Millipore’s Milli-RO Reverse Osmosis System and Milli-Q UV Plus Water System. In addition, all solvents, unless otherwise indicated, were purchased from Fisher and used as received.

5.2.2. Synthesis

The creation of this synergistic peptide ligand is shown in Scheme 5-1. First, the synergistic peptide (PHSRN) is synthesized on the solid support resin (Scheme 5-1A). Second, PEO600 is attached to the N-terminus of this peptide (Scheme 5-1B). Next, the acid functionality of the chain terminus is changed to an amine by attaching ethylene diamine (Scheme 5-1C). Finally, peptide synthesis of the integrin-binding peptide (GSSSGRGDSPA) is performed at the amine of the chain terminus on the solid support resin (Scheme 5-1D), and then 3,6,9-trioxaundecanedioic acid in attached to the N-terminus (Scheme 5-1E).

5.2.2.1. Synthesis of the Synergy Peptide, PHSRN

The synthesis of the PHSRN (Scheme 5-1A) synergy peptide was performed with the aid of an Applied Biosystems 433A peptide synthesizer. The amount of the Fmoc-Knorr-PS resin was 1.0 mmol and the amino acids were reacted at a molar excess of 3 using the FastMoc coupling chemistry protocol with conditional conductivity monitoring of Fmoc deblocking and conditional acetic anhydride capping. After the final Fmoc
Scheme 5-1. Synthesis of two peptides connected by a tether.
deblock the resin was washed with DCM and HPLC grade methanol and dried overnight at room temperature under partial vacuum.

5.2.2.2. Coupling of the PEO600 Tether

The PEO600 tether is attached (Scheme 5-1B) by amide coupling using EDC and NHS. First, the PHSRN peptide on the solid support resin (0.25 mmol) was swelled in 4 mL DMF. PEO600 (2.5 mmol) was mixed with 3 mL DMF. EDC (1 mmol) was dissolved in 1 mL DMF. NHS (1 mmol) was dissolved in 1 mL DMF. The NHS and PEO600 solutions were combined and then added to the swollen resin. Finally, the EDC solution was added dropwise to the reaction flask where the contents were stirred rapidly. The reaction was allowed to stir at 37°C for 16h. Then, three times, the DMF solution was decanted and the resin was washed with DMF.

5.2.2.3. Coupling of EDA

EDA is attached to the PEO600-PHSRN-resin conjugate (Scheme 5-1C) by amide coupling using carbodiimides. First, peptide conjugate on the solid support resin (0.25 mmol) was swelled in 5 mL DMF. EDA (20 mmol) was mixed with 1 mL DMF. EDC (1 mmol) was dissolved in 1 mL DMF and 100 µL of water. The EDA solution was added to the swollen resin. Then, the EDC solution was added dropwise to the reaction flask where the contents were stirred rapidly. The reaction was allowed to stir at 37°C for 3h, after which, dicyclohexylcarbodiimide (1 mmol in 1 mL DMF) was added. The
reaction was allowed to continue for 13h at 37°C. A precipitate was noticed in addition to the solid support resin. Then, three times, the DMF solution was decanted and the resin was washed with DMF.

5.2.2.4. Peptide Synthesis of the Integrin-Binding Peptide

The synthesis of the GSSSGRGDSPA (Scheme 5-1D) integrin-binding peptide was performed with the aid of an Applied Biosystems 433A peptide synthesizer. The EDA-PEO600-PHSRN-resin conjugate (0.25 mmol) were reacted with the amino acids at a molar excess of 3 using the FastMoc coupling chemistry protocol with conditional conductivity monitoring of Fmoc deblocking and conditional acetic anhydride capping. After the final Fmoc deblock the resin was washed with DCM and HPLC grade methanol and dried overnight at room temperature under partial vacuum.

5.2.2.5. Coupling of the PEO220 Spacer

(Scheme 5-1E) First, 3,6,9-trioxaundecanedioic acid (PEO220, 23 mmol) was mixed with DMF (5 mL). The peptide-resin conjugate was swelled in DMF (5 mL). Separately, EDC (1 mmol) was dissolved in DMF (1 mL) and water (100 µL), and NHS (1 mmol), and NHS (1 mmol) was dissolved in DMF (1 mL). The PEO and NHS solutions were combined and then added to the swollen resin. The EDC solution was then added dropwise to the swelled resin and allowed to react under gentle stirring for 16h at 37°C. The reaction solution was decanted from the resulting resin-conjugate,
which was subjected to further washes in DMF (2x), DCM (1x), and methanol (3x) and dried overnight at room temperature under partial vacuum.

5.2.2.6. Cleavage from the Solid Support

The cleavage and deprotection of the peptide-conjugate was performed with a freshly made solution consisting of TFA (86% v), ultra pure water (5% v), phenol (5% v), EDT (3% v), and TIS (1% v) [5.28-30]. (Scheme 5-1F) For every 100 mg of resin, 1.5 mL of the solution was required. The resin, TFA, phenol, water, EDT and TIS were allowed to react under gentle stirring. After four hours, the solution was filtered form the resin. The filtrate was precipitated in cold ethyl ether (ACS grade, 40 mL per 100 mg resin). The filtered polystyrene beads were washed with a few drops of TFA to rinse away any residual peptide. The precipitate was centrifuged (2100 rpm, 3 min.), forming a pellet, and the ether was decanted. Following three additional ether washes, the pellet was dried in a fume hood, dissolved in a small amount of water, and lyophilized. The resulting peptide was characterized via $^1$H-NMR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS).
5.2.3. Characterization

5.2.3.1. NMR

NMR spectra were recorded using a 600 MHz Varian \(^{\text{UNITY INOVA}}\) spectrometer. Samples (7-10 mg) were dissolved in either D\(_2\)O or DMSO-d\(_6\) (680 \(\mu\)L). For 1-d \(^{1}\)H-NMR, 128 scans were collected, and chemical shifts were calibrated to the solvent signal.

5.2.3.2. Mass Spectrometry

A Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) with a Sun Workstation was used to provide molecular weight analysis of peptide conjugates. Analysis via this sensitive technique, which has a mass accuracy between ± 0.01% and ± 0.1%, is useful to distinguish among the desired peptide and its unwanted derivatives. Less than 0.1 mg of the sample was dissolved in ultra pure water (1 mL). The matrix, \(\alpha\)-cyano-4-hydroxycinnamic acid, was dissolved at 25 mg/mL in acetone. The peptide solution (1 \(\mu\)L) and the matrix solution (1 \(\mu\)L) were added together to make a small drop on the metal target plate. 200 scans were collected over a range of 800 m/z – 3600 m/z.
5.2.3.3. Molecular Modeling of Fibronectin and PEO

Molecular modeling analysis was performed with the aid of Insight II (MSI, Inc.) molecular modeling software running on an Octane2 workstation (Silicon Graphics Inc.). First, from the Brookhaven Protein Data Bank (ID#: 1FNF) the molecular coordinates for the crystal structure of fibronectin domains III7, III8, III9, and III10 were downloaded [5.26]. A distance was measured between the RGD, integrin-binding peptide and the PHSRN synergy peptide. This distance between the centers of these two active peptide sequences was found to be 4.4 nm. The distance between the carboxyl terminus of the GRGDSPA sequence and the amino terminus of the PHSRN sequence was measured to be 3.3 nm. This is the distance required to be recreated by the PEO tether. A PEO chain was solvated in water and the chain was allowed to relax using an energy minimization calculation that accounts for solvation energy (Discover module, cvff force field). The molecular weight of the PEO chain was varied until a certain value provided an end-to-end distance of 3.3 nm.

5.3. Results

5.3.1. MALDI-MS of PEO600.

A mass spectrum of the PEO600 from Aldrich was acquired and shown in Figure 5-2. The PEO600 sample had many peaks separated by the weight of one ethylene oxide
Figure 5-2. MALDI-TOF mass spectrum of PEO600. Notice the sample has a binodal molecular distribution centered around 686 (first node) and 1273 (second node).
repeat unit (44), an expected result when dealing with polydisperse samples.

Unexpectedly, however, the sample displayed a bimodal distribution with one
distribution centered on 686 and the other centered on 1273.

5.3.2. Tethered peptide synthesis.

The PHSRN peptide was synthesized on the solid support resin. Each step of the
solid phase peptide synthesis appeared to be sufficient because the synthesizer’s
conductivity monitor showed no step with a markedly lower level of charged species,
which are interpreted as byproducts of deprotection. PEO600 was then coupled to the N-
terminus using EDC and NHS. This was followed by coupling of EDA using EDC and
NHS, and there was a noticeable precipitate as the reaction progressed. Peptide synthesis
of the Integrin-Binding Peptide (GSSSGRGDSPA) then continued, and each step of the
solid phase peptide synthesis appeared to be sufficient because the synthesizer’s
conductivity monitor showed no step with a markedly lower level of charged species.,
The synthesis was completed with the coupling of the PEO220 spacer using EDC and
NHS and, finally, with the cleavage from the solid support using the TFA cocktail.

5.3.2.1. MALDI-MS

After cleavage of the tethered peptide from the resin, MALDI-MS (Figure 5-3)
was used to verify sample composition and purity. The mass spectrum showed three
distributions centered around 1278, 1801, and 2476, where the first two were assigned to
Figure 5-3. MALDI-TOF mass spectrum of the PEO$_{200}$-GSSSGRGDSPA-EDA-PEO$_{600}$-PHSRN sample. The sample also contains PEO$_{600}$-PHSRN. There is a peak at 609 m/z, but since there are other impurities at this low molecular weight and given the successes of conjugating PEOs in the previous chapter, this is most likely another impurity and does not correspond to the PHSRN peptide.
PEO600-PHSRN and the last was assigned to the desired final product—PEO200-GSSSGRGDSPA-EDA-PEO600-PHSRN. Masses were calculated for species in the synthesis: PHSRN, 609; PEO600-PHSRN, 1276, ~1850; EDA-PEO600-PHSRN, 1318, ~1900; GSSSGRGDSPA-EDA-PEO600-PHSRN, 2277, ~2850; PEO200-GSSSGRGDSPA-EDA-PEO600-PHSRN, 2480, ~3050. (Two sets of numbers are quoted for species involving PEO600 due to its bimodal distribution.) There was a peak at 609 m/z, but since there are other impurities at this low molecular weight and given the successes of conjugating PEOs in the previous chapter, this is most likely another impurity and does not correspond to the lone PHSRN peptide.

5.3.2.2. NMR

$^1$H-NMR (Figure 5-4) was also used to verify sample composition and purity. In particular, the downfield segment of the spectrum shows the H$_2$ and H$_3$ aromatic histidine protons at 7.2 and 8.5 ppm. An ethylene oxide peak at 3.55 ppm and the A$_{\beta}$ methyl at 1.2 ppm were also identified. The A$_{\beta}$ methyl peak at 1.2 ppm corresponds to three protons while each of the histidine aromatic protons corresponds to one proton. NMR peak integration was used to compare the amount of alanine to the amount of histidine in the sample with the following calculation: $[A]/[H] = \int_{1.2\text{ppm}} (3\cdot\int_{7.2\text{ppm}})$ or $[A]/[H] = \int_{1.2\text{ppm}} (3\cdot\int_{7.2\text{ppm}})$. $[A]/[H]$ was determined to be 25%.
**Figure 5-4.** 600 MHz $^1$H-NMR spectrum (below) of PEO$_{200}$-GSSSGRGDSP-A-EDA-PEO$_{600}$-PHSRN in deuterium oxide. Enlarged sections of the spectrum (above) highlight the aromatic histidine (H$_5$) protons that only occur in the PHSRN peptide and the alanine methyl (A$\beta$) protons that only occur in the RGD peptide.
5.3.3. Molecular Modeling of Fibronectin and PEO

The crystal structure of fibronectin III$_9$ and III$_{10}$ domains is shown in Figure 5-5. The distance from the arginine of the PHSRN sequence to the arginine of the GRGDSPA sequence was measured to be 4.4 nm. The distance between the carboxyl terminus of the GRGDSPA sequence and the amino terminus of the PHSRN sequence was measured to be 3.3 nm. This is the distance required to be recreated by the PEO tether that joins the two peptide sequences. A PEO chain was solvated in water and the chain was allowed to relax using an energy minimization calculation that accounts for solvation energy (Discover module, cvff force field). The molecular weight of the PEO chain was varied until a certain value provided an end-to-end distance of 3.3 nm; the resulting chain had a molecular weight of 2300 g/mol and is shown in Figure 5-6.

5.4. Discussion

Proposed here is a new peptide ligand for our surfactant polymer biomaterial surface modification to facilitate endothelial cell attachment and proliferation. The surfactant polymer allows for this modification to occur as a simple, one-step dip-coating process. Modifications by the adsorption of amphiphilic molecules onto hydrophobic substrates have been shown previously to be stable even under physiological competitive adsorption and shear conditions [5.31-38]. The designed surfactant polymer consists of a PVAm backbone whose amine functional groups allow for the selective attachment of pendant hydrophobic groups for surface activity and pendant hydrophilic, biologically
Figure 5-5. The crystal structure of FN was used to determine the distance between the N-terminus of the PHSRN peptide and the C-terminus of the GRGDSPA peptide. This is the required end-to-end distance of the tether—3.3 nm.
**Figure 5-6.** One particular conformation from a dynamics simulation of a PEO chain soaked in water. This PEO has a degree of polymerization of 52 (Mw, 2300 g/mol) and provides an end-to-end distance of 3.3 nm.
active peptides. This chapter introduces a new design for a biologically functional hydrophilic pendant group—a PHSRN peptide tethered to an RGD peptide for improved/synergistic binding with EC α5β1 integrins.

Previous attempts have been made to incorporate the synergy peptide into a biomimetic design. Aucoin et al. observed no improved epithelial cell coverage for RGDS-PHSRN (no linker) compared to RGDS alone, while a random surface substitution of the two individual peptides provided an improvement [5.39]. Kim et al. coated polystyrene with the two peptides separated by poly(glycine) spacers (3-12 residues in length), and observed the best osteoblast spreading with the G6 spacer and lesser spreading with the G3, G9 and G12 spacers [5.40]. Benoit et al. used a G13 spacer and observed increased osteoblast density, spreading, and metabolic activity [5.41]. Kao also used a poly(glycine) spacer [5.42]. Both Mardilovich et al. and Ochsenhirt et al. modified a surface using a Langmuir-Blodgett technique and observed positive cellular responses when RGD-PHSRN spacing was at its greatest [5.43, 44]. Mardilovich et al. used a poly(serine-glycine) spacer in order to achieve spacing between the two different peptides, while Ochsenhirt et al. used dilutions of the two different peptide amphiphiles (alkyl-RGD and alkyl-PHSRN) with a third amphiphile (alkyl-PEO) in order to create space between RGD and PHSRN. (It is also noteworthy that Ochsenhirt et al. also created a looped RGD motif in an interesting way by synthesizing a linear RGD sequence as part of a bolaform amphiphile.) Dankers et al. used a random substitution of the two peptides conjugated to the material by supramolecular methods to observe a qualitative increase in fibroblast spreading; however, separation distance of the two peptides was not controlled [5.45]. Petrie et al. and Sharma et al. both used entire recombinant FN III9-
III₁₀ to ensure proper spacing was achieved between the two active sequences [5.46, 47]. Susuki et al. used a commonly available PEO (Mₙ = 3400) as a spacer between the two peptides with no attention paid to how much separation distance was created, and an intermediate amount of cell spreading was observed when compared to a whole FN control (high value) and an RGD peptide control (low value) [5.48].

Our tethered synergy peptide ligand was designed using currently available biochemical data. The GSSSSGRGDSPA peptide sequence was chosen because it is identical to the sequence used in previous studies (Chapters 3 and 4), which will allow for legitimate comparisons to be drawn between the performance of this ligand and the previous one. On the N-terminus of the sequence, a short PEO chain (Mₙ = 220 g /mol) was added as a spacer. This spacer along with the GSSS portion of the peptide sequence will elevate the active portion of the sequence (GRGDSPA) above the surface. The extra elevation is necessary because in FN III₁₀ the RGD loop is 7 -10Å above the rest of the III₁₀ domain [5.26], which enables RGD to fit into the pocket or cleft that is the binding site [5.49]. Attached to the C-terminus of the GSSSSGRGDSPA is a much longer PEO chain, long enough to provide the proper orientation and 30 – 40Å separation between the two peptides [5.26, 27]. Finally, attached to the other end of the long PEO tether is the PHSRN synergy peptide sequence.

To confirm the well-defined synthesis of the peptide tethered construct, it was synthesized on a solid support resin, cleaved and deprotected under acidic conditions. The product was characterized by NMR and mass spectroscopy. The mass spectrum of PEO600 (Figure 5-2) showed two distributions centered on m/z = 686 and the other centered on m/z = 1273. These values were used to calculate the masses of the other
peptide conjugates that were synthesized. The mass spectrum of the final peptide tethered construct (Figure 5-3) showed three distributions centered around $m/z = 1278, 1801, \text{ and } 2476$. The first two were assigned to PEO600-PHSRN and the last was assigned to the desired final product—PEO200-GSSSGRGDSPA-EDA-PEO600-PHSRN. The second, higher distribution for the desired final product ($m/z \sim 3050$) was not readily observed either due to its low concentration or because the spectrum was acquired in linear mode, which tends to increase the relative signal of smaller ($m/z < 3000$) ions over larger ones as opposed to being acquired in reflected mode, which tends to increase the relative signal of larger ions over smaller ones. Information from the NMR spectrum (Figure 5-4) was also used to confirm the structure of the product. $\beta$-methyl protons were observed at 1.2 ppm. This confirms the presence of the GSSSGRGDSPA peptide in the sample, because alanine only occurs in this peptide and not in the PHSRN peptide. This information from the NMR spectrum coupled with the mass of the third distribution observed in the mass spectrum confirms the presence of the desired final product (PEO200-GSSSGRGDSPA-EDA-PEO600-PHSRN). Integration of the NMR spectrum was also used to quantify the purity of the final product. The alanine to histidine ratio was calculated to be $[A]/[H] = 25\%$, meaning that for every one of the desired final product that exists in the sample, there exists three of the PEO600-PHSRN molecules. Two such species in a sample could easily be separated using reversed-phase HPLC using similar methods to the ones that were successfully employed in Chapter 4.

Since the only two species that appeared in the final sample were PEO600-PHSRN and the desired final product (PEO200-GSSSGRGDSPA-EDA-PEO600-PHSRN), it can be concluded that the only difficult step in the total synthesis is the
coupling of EDA. It was evident that every step in the total synthesis leading to the coupling of EDA was successful because the only impurity was PEO600-PHSRN. Also those chains that did happen to be successfully conjugated to EDA proceeded with the rest of the synthesis without any difficulties. The fact that the EDA conjugation was a difficult synthetic step was also confirmed by the precipitate observed during this step. A possible remedy for the difficult EDA conjugation would be to pre-couple Fmoc to one end of EDA. Fmoc-EDA may be more soluble and easier to conjugate to the PEO600-PHSRN on the solid support resin. However, functionalizing just one end of a homobifunctional molecule is not easy and usually involves labor intensive and time consuming chromatographic methods. Fmoc-EDA can be purchased from EMD Biosciences, Inc. (San Diego, CA). Alternatively, in a separate vessel before coupling it to the solid support resin, EDA can be reacted with Fmoc-oxysuccinimide in an 10x : 18x (EDA : Fmoc) ratio, which would statistically yield Fmoc-EDA-Fmoc (81%), Fmoc-EDA (18%), and EDA (1%). This statistical yield could then be added to the PEO600-PHSRN on the resin along with EDC and NHS. Then after multiple washings, peptide synthesis could proceed with the GSSSRGDSG peptide.

PEO600 was used as the tether as a proof of concept for the synthetic design. As a random coil in solution, it is much too small to create the required 3.3 nm separation between the proline amino group and the alanine carboxylate. The molecular dimensions of PEO in aqueous solutions has been deduced by intrinsic viscosity determination [5.50-53], small angle neutron scattering [5.54], light scattering [5.55], and molecular simulations [5.56]. The values determined from viscometry, light scattering, and small angle neutron scattering were plotted in Figure 5-7. The data was visually interpolated to
Figure 5-7. Plot mean-square end-to-end distance versus molecular weight for PEO in aqueous solution. Values from various studies were sampled: Armstrong et al. [5.51], Atha and Ingham [5.52], Amu [5.50] Chew and Couper [5.53], Devanand and Selser [5.55], and Vennemann et al. [5.54]. A point on the graph was also plotted at (M_n = 1250 g/mol, <R^2>^{1/2} = 3.3 nm).
estimate that a 1250 g/mol molecular weight would provide an end-to-end distance of 3.3 nm. Samoria and Blankschtein’s rotational isomeric state simulation of PEO in aqueous solution predicts that a 2400 g/mol molecular weight would provide an end-to-end distance of 3.3 nm [5.56]. All of these methods do have their shortcomings as the end-to-end distance is not experimentally measured directly and must either be theoretically calculated for experimental observations or derived from molecular simulations based on observed thermodynamic data.

The polydispersity of PEO600 is quite evident in Figure 5-2. This result was expected considering that all commercially available PEOs with a molecular weight of 600 or higher are polydisperse. The polydispersity of PEO creates an interesting problem when attempting to create a tether between the integrin and synergy peptide. Each molecular weight fraction corresponds to a chain with different degree of polymerization, and hence, a different chain length. The root-mean-square end-to-end distance of a polymer chain, \(<R^2>^{1/2}\), can be described as [5.57, 58]

$$< R^2 >^{1/2} = aN^\nu$$

where a is sometimes thought of as the bond length, N is the number of bonds (3 times the degree of polymerization for PEO), and \(\nu\) is an exponent that depends on solvents conditions (\(\nu = 0.6\) in a good solvent, \(\nu = 0.5\) under \(\theta\)-solvent conditions). Therefore, chains of different degrees of polymerization will also have different end-to-end distances, and a polydisperse sample of PEO contains chains with many differing end-to-end distances. In the case of biological ligand-receptor binding, there is no tolerance for a polydispersity of end-to-end distances because the arginine of the synergy peptide must
be 4.4 nm from the arginine of the integrin-binding peptide in order to ensure synergistic binding.

Solid phase synthetic methods can be employed to create a monodisperse PEO-based tether. An Fmoc-protected PEO-based amino acid must be created as shown in Scheme 5-2. First, 3,6,9-trioxaundecanedioic acid (in excess) is coupled to a Wang resin using HBTU as the activator, HOBt as a catalyst, and DIEA as a proton scavenger (Scheme 5-2(A)). Next, EDA is coupled to the free acid end using DIC and NHS (Scheme 5-2(B)). (If EDA coupling is difficult as observed before, then Fmoc can be pre-coupled as outlined above.) Then, Fmoc-oxysuccinimide is coupled to the free primary amine (Scheme 5-2(C)). Finally, cleavage from the resin is performed using TFA and water (95:5 (v:v)) to yield the Fmoc-protected PEO-based amino acid, Fmoc-pegaa (Scheme 5-2(D)). A similar strategy for converting a homobifunctional molecule to a heterobifunctional molecule was also proposed by Leznoff and Wong [5.59]. Here, 1,4 butanediol (in excess) was attached to a solid support resin, and the free alcohol was protected with a tosyl group. The yield of loading was 10% and the product was 50% monoprotected and 50% unprotected. Today’s protecting groups (esp. Fmoc) and activators (esp. HBTU) would definitely provide better yields.

Fmoc-pegaa can be used like any standard protected amino acid is used during solid phase synthesis. As shown in Scheme 5-3(A), and peptide can be synthesized using any number of Fmoc-pegaa to serve as the tether between the integrin-binding sequence and the synergy sequence. Next, as done before in this chapter and the previous one, 3,6,9-trioxaundecanedioic acid (in excess) is coupled to the amine terminus of the peptide (Scheme 5-3(B)). Cleavage and deprotection (except for the –Obzl group) is performed
Scheme 5-3. Synthesis of two peptides connected by a tether using PEG<sub>aa</sub>.
using the standard 85% TFA cocktail (Scheme 5-3(C)). Then, the peptide-tether
construct is coupled to poly(vinyl amine) using EDC and NHS, and in the following step,
an alkyl acid is also coupled to the poly(vinyl amine) using EDC and NHS (Scheme 5-
3(D)). (The length of the alkyl acid can be chosen using the concepts in the previous
chapter.) Finally, aspartic acid benzyl protection is removed using
trifluoromethanesulfonic acid.

The pegaa provides a convenient way to study the effect differing tether lengths
has on integrin affinity: Tethers could be synthesized with end-to-end distances that
would space the intergin-binding sequence at distances (d) d < 4.4 nm, d > 4.4 nm, d <<
4.4 nm, d >> 4.4 nm, or even d = 4.4 nm from the synergy sequence. However, the end-
to-end distances quoted in the literature will no longer be valid for this new tether, since
with the addition of the amide functionality, the tether now has a new and unique
chemical structure.

In order for a ligand to bind a receptor, it must be in the proper position in three-
dimensional space, and in the proper orientation. For the ligand to be in the proper
position, there are three translational degrees of freedom, one for translation along each
of the Cartesian axes (x, y, z), or in the case of spherical coordinates (ρ, θ, φ). Since
peptides are chiral molecules, there is no symmetry, and for the ligand to be in the proper
orientation, there are also three rotational degrees of freedom, one for rotation around
each Cartesian axis (x, y, z). For complete binding of FN to α5β1, the synergy peptide-
sequence and the integrin-binding sequence must also be in the proper position and
orientation relative to each other. Therefore, there are six degrees of freedom of the
synergy peptide with respect to the integrin-biding peptide. However, within FN, the
structure of the type III domains restricts the position and orientation of the two peptides relative to each other. The solution NMR structure of FNIII9-10 showed the domains are very rigid, but there is flexibility between the two domains [5.60]. This flexibility has been described as a hinge, which would correspond to one degree of freedom; however, this hinge-like behavior could result from rotation around a few, \( n \), amino acid dihedral angles (\( \varphi \), \( \psi \)), which is almost like \( n \) degrees of freedom.

This smaller number in degrees of freedom between FN III9 and FN III10 is much less than the six degrees of freedom that independent integrin-binding and synergy peptides dissolved in solution have. The amount of degrees of freedom translates to amount of entropy. Upon binding of the two independent peptides in solution to the \( \alpha_5 \beta_1 \) integrin the six degrees of freedom of the two peptides relative to each other are restricted, which corresponds to a large entropic cost. This observation that the two independent peptides in solution do not exhibit synergistic behavior [5.25] fortifies the concept of large entropic cost. (Synergistic binding is defined here as affinity of binding that is much, much greater (sometimes orders of magnitude greater) than the sum of the affinities of each of the independent ligands.) For FN III9-10, which has fewer degrees of freedom of the synergy sequence relative to the integrin-binding sequence, to bind the \( \alpha_5 \beta_1 \) integrin, fewer degrees of freedom are needed to be restricted in order for both sequences to bind, which corresponds to a smaller entropic cost, resulting in synergistic binding. In other words, if one peptide sequence in FN III9-10 binds, the other is already held in almost the proper position and orientation, so it can bind at a very small entropic cost.
Our design consists of holding the PHSRN sequence away from the RGD sequence using a flexible tether. This is the restriction of the \( \rho \) coordinate in spherical coordinates. There are still two translational and three orientational degrees of freedom. The tether is also a flexible random coil, so \( \rho \) is not held to a fixed value, but a range of values. The observed value of binding for our design should be somewhere between that of two independent peptides in solution and that of the synergistic binding of FN to the integrin. An evolution in the design may be to have a rigid bridge separating the two peptides instead of a flexible tether. A rigid bridge, like a peptide based \( \alpha \)-helix, a liquid crystal type segment, or a ladder type polymer chain, would further restrict the degrees of freedom between the two peptides. Altroff \textit{et al.} has proposed that there is a structural stability requirement for proper binding of FN to the integrin \cite{5.61, 62}, and this structural stability may also be required for PHSRN/RGD peptide systems.

When it comes time to couple our tethered ligand to PVAm, it probably would be desirable to not load the ligand onto the PVAm at maximum capacity. The integrin has a pocket or cleft into which the RGD sequence fits \cite{5.49}; therefore the RGD sequence must be extended above the surface in order to bind with the integrin \cite{5.26}. It is desirable for the tethered ligand to be extended above other surface elements at a less than maximal density so room exists for the integrin’s pocket to fit around the ligand.
5.5. Conclusion

It is hypothesized that a material whose surface possesses both the integrin-binding and the synergy peptides separated at the proper distance and in the proper orientation would bind the $\alpha_5\beta_1$ integrin on EC, thus promoting endothelialization and leading to a nonthrombogenic surface. This study provides a proof of concept for creating a PEO tether between PHSRN and RGD and characterizing this tethered ligand. This PHSRN-PEO-RGD molecule was synthesized, and ground work is laid for making a monodisperse tether. The tethered ligand could be used to interact with the $\alpha_5\beta_1$ integrin by varying the length of the tether and possibly be used to produce nonthrombogenic, endothelialized cardiovascular biomaterials.

5.6. Acknowledgments

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5.7. References


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Chapter 6. DESIGN, SYNTHESIS, AND CHARACTERIZATION OF A HIGH AFFINITY CYCLIC PEPTIDE LIGAND

6.1. Introduction

In the efforts to create a non-thrombogenic vascular graft, perhaps the most promising is to modify the surface of a current graft material to support a layer of vascular endothelium on the interior of the graft. The endothelium, composed of endothelial cells (EC), is the natural lining of blood vessels, and therefore has an inherent resistance to thrombosis [6.1-7]. However, human EC do not adhere to the hydrophobic surfaces of polymer grafts [6.8-12]. An interface must be created that both adheres to the polymer surface and supports EC attachment, growth, and proliferation. These interfaces usually incorporate the cell adhesive peptide, arginine-glycine-aspartic acid (RGD), which is a native peptide sequence found in many extracellular matrix (ECM) proteins, including fibronectin (FN) where it binds with the EC’s α5β1 integrin. One problem is that RGD cannot completely mimic FN’s ability to bind to EC integrin [6.13-15].

It has been shown that RGD sequence in FN III10 is part of a tightly constrained loop in the peptide backbone that extends 7 -10 Å out from the surface of the rest of FN [6.16]. However short, linear peptides cannot mimic this looped conformation because these peptides have too much conformational freedom [6.17]. It has been hypothesized that by limiting the conformational freedom of these linear peptides by forming a cyclic peptide, called a lactam, the affinity of the peptide for the integrins can be increased
A cyclic RGD lactam bends the peptide backbone at the R-G-D sequence, and is thus a better mimic of the native RGD loop conformation. Kessler’s group first discovered, by generating a small peptide library, that a particular RGD containing 5-membered cyclic lactam has particularly high biological activity (> 100 fold improvement in affinity over linear peptides) [6.19, 20]. This peptide was cyclic(RGDfV), where “f” denotes D-phenylalanine instead of the naturally occurring, L-phenylalanine. However, this peptide is not easily conjugated to materials, because the f and V amino acids do not contain useful functional group for bioconjugation, and conjugation via the R, G, or D residues would render the peptide biologically inactive. It was then determined that the amino acid in valine’s position was not important for bioactivity [6.21]. Therefore, a lysine could now be placed in valine’s position to allow for easy bioconjugation through the ε-amino group with no ill effects on bioactivity. Therefore, incorporation of this cyclic RGD peptide, c(RGDfK) into our biomimetic design should enhance the affinity to ECs to our material surfaces through attachments made by the αvβ3 integrins.

A design of a vascular graft surface modification that will promote endothelialization is proposed. It is an amphiphilic polymer, which will modify a graft through a simple dip-coating process (Figure 6-1). This polymer design consists of a poly(vinyl amine) (PVAm) backbone with hydrophobic alkyl branches for adsorption onto the material surface. The hydrophilic groups consist of an RGD containing cyclic lactam peptide. The cyclic nature of the RGD peptide is a better mimic of the RGD loop in FN as opposed to the linear RGD peptide. Incorporation of this cell adhesive peptide should result in increased cell receptor affinity and an increased cell response. In this
Figure 6-1. Design of a biomimetic surfactant polymer that encourages high-affinity binding to EC integrins. Attached to a PVAm backbone (A) are hydrophobic legs (B) for adsorption onto the material surface and integrin-binding, RGD-containing cyclic peptides (C).
study, the cyclic RGD hydrophile is synthesized and characterized using mass
spectrometry and nuclear magnetic resonance spectroscopy.

6.2. Materials and Methods

6.2.1. Materials

For solid phase peptide synthesis (SPPS) all protected and unprotected amino
acids (Fmoc-Arg(Pbf)-OH, Fmoc-Asp-OAll, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-
Glu-OAll, Fmoc-Lys(Boc)-OH, Fmoc-D-Phe-OH, and Fmoc-Ser(tBu)-OH) were
purchased from Anaspec, Inc. (San Jose, CA). The N, N-dimethylformamide (DMF), N-
methylpyrrolidone (NMP), dichloromethane (DCM), and N, N-diisopropylethylamine
(DIPEA) solvents were SPPS grade and purchased from Applied Biosystems (Foster
City, CA). The Knorr-polystyrene solid support resin (Fmoc-Knorr-PS) the Wang resin,
the activating agent cocktail, HBTU ((2-(1H-benzotriazol-1-yl)1,1,3,3-
tetramethylyuronium hexafluorophosphate) with HOBr/DMF (0.5 M 1-
hydroxybenzotriazole in N, N-dimethylformamide), and the cleavage/deprotection
reagent, HATU (N-[(dimethyamino)-1H-1, 2, 3-triazolo[4, 5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide), trifluoroacetic acid (TFA),
were also purchased from Applied Biosystems. The scavengers for the peptide cleavage
reaction (phenol, 1,2-ethanediethiol (EDT), and triisopropylsilane (TIS)) along with
piperidine, N-methylmorpholine (NMM), and tetrakis(triphenylphosphine)palladium(0)
(Pd(PPh₃)₄), and dicyclohexyl carbodiimide (DCC) were purchased from Aldrich. All SPPS and peptide cleavage solvents and reagents were used as received.

In the nuclear magnetic resonance spectroscopy experiments deuterated water (D₂O) and dimethyl-d₆ sulfoxide (DMSO-d₆) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Also, a-cyano-4-hydroxycinnamic acid was obtained from Aldrich as the MALDI-MS matrix.

Ultra pure water was produced by Millipore’s Milli-RO Reverse Osmosis System and Milli-Q UV Plus Water System. In addition, all solvents, unless otherwise indicated, were purchased from Fisher and used as received.

6.2.2. c(RGDfK) Synthesis

The c(RGDfK) peptide was synthesized as a proof of synthetic concept, the synthesized peptide could also be used in cell-growth inhibition studies. It was first synthesized based on the method of McCusker et al. [6.22] (Scheme 6-1). Briefly, on the ABI 433a peptide synthesizer Fmoc-Asp-OAll was coupled through its γ-carboxylate to the Wang resin using DCC and dimethyl amino pyridine (Scheme 6-1(A)). Peptide synthesis then followed with the sequential Fmoc deprotection and the coupling of Fmoc-Gly-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(Boc)-OH, and finally Fmoc-D-Phe-OH (Scheme 6-1(B)). Next, the C-terminal –OAll group was removed (Pd(PPh₃)₄ (3equiv.), chloroform-acetic acid-NMM (37:2:1), room temperature (rt), 2 h) (Scheme 6-1(C)). The N-terminal Fmoc group was then removed using piperidine and the ABI 433a (Scheme 6-1(D)). Then, the cyclization was performed (HATU (2 equiv), DIPEA (4 equiv.), DMF,
Scheme 6-1. c(RGDfK) synthesis.
Scheme 6-1 (cont.). c(RGDK) synthesis.
rt, 16 h) (Scheme 6-1(E)). Finally, the cyclic peptide was cleaved from the resin and deprotected using an 85% TFA cocktail (Scheme 6-1(F)).

c(RDGfK) was also synthesized again, but with a few modifications. The synthesis proceeded as above, but allylic deprotection was performed on the ABI 433a synthesizer and more extensive washing procedures were used to better remove any traces of Pd(PPh₃)₄ and acetic acid. The resin was washed with chloroform (3x), then the deprotection was performed as above but for 4 h. The resin was washed again (chloroform, 3x), and the deprotection was applied again for 12 h. The resin was then washed multiple times: DCM (3x), 0.46 M DIPEA and 0.02 M sodium diethyllditiocarbamate in DMF (3x), DMF (3x), 0.46 M DIPEA in DCM (3x), and DMF (3x). Also, the cyclization procedure using HATU was performed for only 1 h, not 16 h as above.

6.2.3. Synthesis c(RGDfE(SSSK))

The c(RGDfE(SSSK)) peptide was synthesized (Scheme 6-2) for incorporation into our PVAm-based surfactant polymers where glutaric dialdehyde serves as a linker between the peptide and the polymer as in Chapter 3. On the ABI 433a peptide synthesizer, using the Knorr resin, peptide synthesis followed with the sequential Fmoc deprotection and the coupling of Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Ser(tBu)-OH (Scheme 6-2(A)). Fmoc-Glu-OAll was then coupled through its δ-carboxylate propagating peptide’s N-terminus (Scheme 6-2(B)). Peptide synthesis then followed with the sequential Fmoc deprotection and the coupling of Fmoc-
Scheme 6-2. c(RGfE(SSSK)) synthesis.
Scheme 6-2 (cont.) c(RGDfE(SSSK)) synthesis.
D-Phe-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, and finally Fmoc-Arg(Pbf)-OH (Scheme 6-2(C)). The resin was washed with chloroform (3x). Next, the α-allyl ester of glutamate was removed (Pd(PPh₃)₄ (3 equiv.), chloroform-acetic acid-NMM (37:2:1), rt, 4 h). The resin was washed again (chloroform, 3x), and the allyl ester deprotection was applied again for 12 h. (Scheme 6-2(D)). The resin was then washed multiple times: DCM (3x), 0.46 M DIEA and 0.02 M sodium diethyldithiocarbamate in DMF (3x), DMF (3x), 0.46 M DIPEA in DCM (3x), and DMF (3x). The N-terminal Fmoc group was then removed using piperidine and the ABI 433a (Scheme 6-2(E)). Then, the cyclization was performed (HATU (2 equiv), DIPEA (4 equiv.), DMF, rt, 1 h) (Scheme 6-2(F)). Finally, the cyclic peptide was cleaved from the resin and deprotected using an 85% TFA cocktail (Scheme 6-2(G)).

6.2.4. Synthesis of Fmoc-Lys-OAll

A modified amino acid, Fmoc-Lys-OAll, was synthesized (Scheme 6-3) to enable the future synthesis of c(RGDfK(PEO₂₂₀)), which, instead of using glutaric dialdehyde, uses a small PEO molecule, as in Chapter 4, as the linker between the peptide and the PVAm surfactant polymer backbone. Synthesis was adapted from Andreau et al. [6.23]. Fmoc-Lys(Boc)-OH (16 mmol), allyl bromide (15.2 mL), acetonitrile (15.2 mL), and DIPEA (32 mmol) were stirred and heated at 40°C for 5 h (Scheme 6-3(A)). Ethyl acetate (275 mL) was added, and the product, in ethyl acetate solution, was washed with 175 mL of 0.1 N hydrochloric acid (4x), 175 mL of 1.2 M aqueous sodium bicarbonate (4x), and 175 mL of saturated sodium chloride aqueous solution (4x). The product, in
Scheme 6-3. Fmoc-Lys-OAll synthesis.
ethyl acetate, was then dried over magnesium sulfate, centrifuged, and then decanted. The solvent was removed by rotary evaporation. Product: Fmoc-Lys(Boc)-OAll, 7.95 g, 15.6 mmol, 97.7% yield. Product was characterized using $^1$H-NMR and analytical scale rp-HPLC.

Boc deprotection (Scheme 6-3(B)) was adapted from Kappel et al. [6.24]. Fmoc-Lys(Boc)-OAll (15 mmol) was stirred for 1 h at rt in TFA : water (95 : 5, v : v). The product was precipitated in cold ethyl ether (1 L), and centrifuged. The solvent was decanted and the product was dried in a vacuum oven overnight at rt. The product, Fmoc-Lys-OAll, was then separated from the impurity, Fmoc-Lys-OH, using preparative scale rp-HPLC. Product was characterized using $^1$H-NMR and analytical scale rp-HPLC.

6.2.5. Characterization

6.2.5.1. NMR

NMR spectra were recorded using a 600 MHz Varian $^\text{UNITY}$NOVA spectrometer. Samples (7-10 mg) were dissolved in either D$_2$O or DMSO-d$_6$ (680 μL). For 1-d $^1$H-NMR, 128 scans were collected, and chemical shifts were calibrated to the solvent signal.

6.2.5.2. Mass Spectrometry

A Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) with a Sun Workstation was used to provide molecular
weight analysis of peptide conjugates. Analysis via this sensitive technique, which has a mass accuracy between ± 0.01% and ± 0.1%, is useful to distinguish among the desired peptide and its unwanted derivatives. Less than 0.1 mg of the sample was dissolved in ultra pure water (1 mL). The matrix, α-cyano-4-hydroxycinnamic acid, was dissolved at 25 mg/mL in acetone. The peptide solution (1 μL) and the matrix solution (1 μL) were added together to make a small drop on the metal target plate. 200 scans were collected over a range of 800 m/z – 3600 m/z.

6.2.5.3. rp-HPLC

For reversed-phase HPLC purification of PEO-HBP1, a Waters 2690 separations module was used along with a Waters 2487 UV absorbance detector controlled by the Millennium software (v. 2.0). Peptide or amino acid (less than 100 mg) was dissolved in no more than 2 mL of the mobile phase, 0.1% TFA in ultra pure water, and loaded onto an Alltech Hyperprep PEP 100Å 8 μ (22 mm × 250 mm) preparatory scale C-18 column. For the purification of c(RGDfK), linear gradients of 0.1% aqueous TFA and 0.082% TFA in acetonitrile were run at 5.0 mL min⁻¹ flow rate from 98:2 over 5 minutes, then 98:2 to 85:15 for 5 min, then 85:15 to 75:25 for 95 min, and then 75:25 to 2:98 for 20 min. UV absorbance was monitored at 220 nm and 254 nm, wavelengths specific for the peptide bond and the tryptophan residue, respectively, and the c(RGDfK) fraction was collected 31-37 minutes. For the purification of c(RGDfE(SSSK)), linear gradients of 0.1% aqueous TFA and 0.082% TFA in acetonitrile were run at 5.0 mL min⁻¹ flow rate from 98:2 over 5 minutes, then 98:2 to 86:14 for 5 min, then 86:14 to 76:24 for 95 min,
and then 76:24 to 2:98 for 20 min. UV absorbance was monitored at 220 nm and 254 nm, wavelengths specific for the peptide bond and the tryptophan residue, respectively, and the c(RGDfE(SSSK)) fraction was collected 26-34 minutes. For the purification of the Fmoc-lys-OAll amino acid, linear gradients of 0.1% aqueous TFA and 0.082% TFA in acetonitrile were run at 5.0 mL min⁻¹ flow rate from 98:2 over 5 minutes, then 98:2 to 61:39 for 5 min, then 61:39 to 60.4:39.6 for 15 min, then 60.4:39.6 to 58.8:41.2 for 1 min, then 58.8:41.2 to 57.5:42.5 for 54 min, and then 57.5:42.5 to 2:98 for 20 min. UV absorbance was monitored at 220 nm and 280 nm, wavelengths specific for the urethane bond and the fluorenyl group, respectively. The Fmoc-lys-OAll fraction was collected 39-80 min, and the Fmoc-lys-OH fraction was collected from 29-39 min. For the analysis of the protected lysine amino acids, linear gradients of 0.1% aqueous TFA and 0.082% TFA in acetonitrile were run using a Beckman Ultrasphere 5μ (4.6 mm × 250 mm) C-18 column at 1.0 mL min⁻¹ flow rate from 90:10 to 0:100 over 30 minutes, then 0:100 for 5 min. UV absorbance was monitored at 220 nm and 280 nm, wavelengths specific for the urethane bond and the fluorenyl group, respectively.
6.3. Results

6.3.1. Synthesis and Characterization of c(RGDfK)

6.3.1.1. Synthesis

The c(RGDfK) peptide was synthesized on the solid support resin. Each coupling and Fmoc deprotection of the solid phase peptide synthesis appeared to be sufficient because the synthesizer’s conductivity monitor showed no step with a markedly lower level of charged species, which are interpreted as byproducts of deprotection. The allylic deprotection was complete; however, the resin was stained a grayish color. Subsequent Fmoc deprotection was completed with favorable conductivity measurements. After 16 h exposure to HATU during the cyclization reaction, the reaction mixture and the resin was stained dark reddish-brown. The synthesis was completed, finally, with the cleavage from the solid support using the TFA cocktail. Using the second synthesis protocol, the extra washing procedure prevented the staining of the resin after allylic deprotection, and less of a color change of the reaction mixture and staining of the resin was noticed as a result of the shorter cyclization reaction.

6.3.1.2. MALDI-TOF Mass Spectrometry

After cleavage of c(RGDfK) from the resin, MALDI-MS (Figures 6-2 and 6-3) was used to verify sample composition and purity. The mass spectrum of c(RGDfK)
Figure 6-2. MALDI-TOF mass spectrum of c(RGDfK) made using the first synthetic method. There is no peak of the desired product at 604.3 m/z. There is a peak at 619 m/z, but this is not the desired product.
Figure 6-3. MALDI-TOF mass spectrum of steps in the second synthetic method to make c(RGDfK). (All spectra are of crude product unless otherwise specified.) (A) Fmoc-fKRGD-OAll peak observed at 885 m/z (884.42 calculated). (B) Fmoc-fKRGD-OH peak observed at 844 m/z (844.39 calculated). (C) H-fKRGD-OH peak observed at 623 m/z (622.32 calculated). (D) c(RGDfK) with peak observed at 605 m/z (604.31 calculated) along with an additional peak observed at 619 m/z. (E) rp-HPLC purified product of c(RGDIK) with peak observed at 605 m/z (604.31 calculated).
made using the first synthetic method (Figure 6-2) showed a peak at 618.8 m/z, an
undesirable product instead of the desired product with a calculated m/z of 604.3. Mass
spectra were acquired for each step of the second synthetic method (Figure 6-3). Figure
6-3(A) shows Fmoc-fKRGD-OAll, m/z = 885 (884.42 calculated). Figure 6-3(B) shows
Fmoc-fKRGD-OH, m/z = 844 (844.39 calculated). Figure 6-3(C) shows H-fKRGD-OH,
m/z = 623 (622.32 calculated). Figure 6-3(D) shows the crude product of c(RGDfK), m/z
= 604 (604.31 calculated) along with an additional peak observed at 619 m/z. Figure 6-
3(E) shows rp-HPLC purified c(RGDfK), m/z = 605 (604.31 calculated).

6.3.1.3. 1H-NMR

1H-NMR (Figure 6-4) in deuterium oxide was used to verify sample composition
and purity. The peaks were assigned as follows: chemical shift in ppm (peak splitting,
amino acid assignment, number of protons), δ 7.15 (d, fζ, 2H), δ 7.09 (t, fζ, 1H), δ 7.02 (d,
fδ, 2H), δ 4.38 (m, Dα, 1H), δ 4.30 (m, fε, 1H), δ 4.21 (dd, Rα, 1H), δ 4.01 (d, Gα, 1H), δ
3.61 (dd, Kα, 1H), δ 3.39 (d, Gα, 1H), δ 2.94 (t, Rβ, 2H), δ 2.88 (dd, fβ, 1H), δ 2.60-2.72
(m, Kε, fβ, 3H), δ 2.55 (m, Dβ, 1H), δ 2.47 (s, Dβ, 1H), δ 1.74 (m, Rβ, 1H), δ 1.38-1.58 (m,
Rβ, Kβ, 2H), δ 1.10-1.38 (m, Kβ, Kγ, Rγ, 5H), and δ 0.44-0.76 (m, Kδ, 2H).

6.3.1.4. rp-HPLC

An analytical scale rp-HPLC chromatogram of the crude product of c(RGDfK) is
shown in Figure 6-5(A). c(RGDfK) elutes at 35.8 min, and the undesirable product with
Figure 6-4. 600 MHz $^1$H-NMR spectrum of c(RGDfK) in deuterium oxide.
Figure 6-5. (A) Analytical scale rp-HPLC chromatogram of the crude product of c(RGDfK). The integrated ratio of the desired product to the undesirable product with an observed m/z of 619 was 1:0.82 for the 220 nm wavelength and 1:1.75 for 254 nm. (B) rp-HPLC chromatogram of c(RGDfK) purification. The collected fraction was from 31-37 min.
an observed m/z of 619 elutes at 44.2 min. The integrated ratio of the desired product to the undesirable product with an observed m/z of 619 was 1:0.82 for the 220 nm wavelength and 1:1.75 for 254 nm. rp-HPLC chromatogram of c(RGDfK) purification is shown in Figure 6-5(B). The collected fraction was from 31-37 min.

6.3.2. Synthesis and Characterization of c(RGDfE(SSSK))

6.3.2.1. Synthesis

The c(RGDfE(SSSK)) peptide was synthesized on the solid support resin. Each coupling and Fmoc deprotection of the solid phase peptide synthesis appeared to be sufficient because the synthesizer’s conductivity monitor showed no step with a markedly lower level of charged species, which are interpreted as byproducts of deprotection. Cyclization was performed with 1 h exposure to HATU. The synthesis was completed, finally, with the cleavage from the solid support using the TFA cocktail.

6.3.2.2. rp-HPLC

An analytical scale rp-HPLC chromatogram of the crude product of c(RGDfE(SSSK)) is shown in Figure 6-6(A). c(RGDfE(SSSK)) elutes at 33.5 min, and an undesirable product elutes at 46.5 min. The integrated ratio of the desired product to the undesirable product was 1:0.25 for the 220 nm wavelength and 1:0.39 for 254 nm.
Figure 6-6. (A) Analytical scale rp-HPLC chromatogram of the crude product of c(RGDfE(SSSK)). The integrated ratio of the desired product to the undesirable product with an observed m/z of [M+H+14]+ was 1:0.25 for the 220 nm wavelength and 1:0.39 for 254 nm. (B) rp-HPLC chromatogram of c(RGDfE(SSSK)) purification. The collected fraction was from 26-34 min.
rp-HPLC chromatogram of c(RGDfE(SSSK)) purification is shown in Figure 6-6(B). The collected fraction was from 26-34 min.

6.3.2.3. MALDI-TOF Mass Spectrometry

After cleavage of c(RGDfE(SSSK)) from the resin and purification by rp-HPLC, MALDI-MS (Figure 6-7) was used to verify sample composition and purity. The spectrum shows rp-HPLC purified c(RGDfE(SSSK)), m/z = 993 (993.47 calculated).

6.3.3. Synthesis and Characterization of Fmoc-Lys-OAll

6.3.3.1. Synthesis

The Fmoc-lys-OAll amino acid was synthesized by first protecting the carboxylate of Fmoc-lys(Boc)-OH with allyl bromide, followed by Boc deprotection using TFA. The synthesized amino acid will be used in the future synthesis of a cyclic RGD peptide with a PEO spacer.

6.3.3.3. rp-HPLC

The lysine starting material, Fmoc-lys(Boc)-OH; the synthetic intermediate, Fmoc-lys(Boc)-OAll; the product, Fmoc-lys-OAll; and the side product, Fmoc-lys-OH were compared using analytical rp-HPLC (Figure 6-8A). Fmoc-lys(Boc)-OH had a
Figure 6-7. rp-HPLC purified c(RGDfE(SSSK)) with peak observed at 993 m/z (993.47 calculated).
Figure 6-8. (A) Analytical scale rp-HPLC chromatograms of protected lysines monitored at 220 nm (urethane bonds) and 280 nm (flourenyl group). (B) rp-HPLC chromatogram of lysine purification. Fmoc-lys-OAll was collected from 40-70 min., and Fmoc-lys-OH was collected from 31-37 min.
retention time of 18.7 min. Fmoc-lys(Boc)-OAll had a markedly different retention time of 22.4 min. Fmoc-lys-OH had a markedly different retention time of 12.3 min. Fmoc-lys-OAll had a markedly different retention time of 15.0 min. The integration of both wavelengths of the chromatograms of these peaks at the four different retention times was used to estimate purity (Table 6-1).

The rp-HPLC chromatogram of Fmoc-lys-OAll purification is shown in Figure 6-8B. Fmoc-lys-OAll was collected from 40-70 min., and Fmoc-lys-OH was collected from 31-37 min.

6.3.3.4. 1H-NMR

1H-NMR of Fmoc-lys-OAll in deuterated methanol was used to confirm sample composition and purity (Figure 6-9). The peaks were assigned as follows: chemical shift in ppm (peak splitting, amino acid assignment, number of protons), δ 7.79 (d, fluorenyl, 2H), δ 7.66 (dd, fluorenyl, 2H), δ 7.38 (t, fluorenyl, 2H), δ 7.30 (t, fluorenyl, 2H), δ 5.92 (m, vinyl, 1H), δ 5.31 (m, vinyl, 1H), δ 5.20 (m, vinyl, 1H), δ 4.61 (d, allyl methylene, 2H), δ 4.41 (dd, methine, 1H), δ 4.32 (dd, methine, 1H), δ 4.21 (m, Fmoc methylene, 2H), δ 2.89 (m, Kε, 2H), δ 1.88 (m, Kβ, 1H), δ 1.71 (m, Kβ, 1H), δ 1.64 (m, Kδ, 2H), and δ 1.46 (m, Kγ, 2H). Enlarged sections of the spectrum (Figure 6-10 and 6-11) are compared to the other protected lysine derivatives in the vicinity of 5 ppm and 1 ppm to highlight the key protecting groups, OAll and Boc, respectively. Most notably, Fmoc-lys-OAll has the allylic protons in the vicinity 5 ppm and lacks the methyl singlet of the Boc protecting group at 0.8 ppm.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Component (%)</th>
<th>220 nm</th>
<th>280 nm</th>
</tr>
</thead>
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<tr>
<td></td>
<td>lys(Boc)-OH</td>
<td>lys(Boc)-OAll</td>
<td>lys-OH</td>
</tr>
<tr>
<td>lys(Boc)-OH</td>
<td>94.54</td>
<td>4.81</td>
<td>0.62</td>
</tr>
<tr>
<td>lys(Boc)-OAll</td>
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<td>95.47</td>
<td>0.00</td>
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<tr>
<td>crude product</td>
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<td>1.42</td>
<td>27.56</td>
</tr>
<tr>
<td>lys-OH</td>
<td></td>
<td>99.81</td>
<td>0.19</td>
</tr>
<tr>
<td>lys-OAll</td>
<td>0.06</td>
<td>3.91</td>
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<table>
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<th>Sample Name</th>
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<th>280 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lys(Boc)-OH</td>
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<td>lys-OH</td>
</tr>
<tr>
<td>lys(Boc)-OH</td>
<td>96.01</td>
<td>3.26</td>
<td>0.66</td>
</tr>
<tr>
<td>lys(Boc)-OAll</td>
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<td>97.84</td>
<td>0.00</td>
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<td>0.81</td>
<td>27.62</td>
</tr>
<tr>
<td>lys-OH</td>
<td></td>
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<td>0.15</td>
</tr>
<tr>
<td>lys-OAll</td>
<td>0.03</td>
<td>3.38</td>
<td></td>
</tr>
</tbody>
</table>

Table 6-1. Protected amino acid sample composition as determined by rp-HPLC peak integration.
Figure 6-9. 600 MHz $^1$H-NMR spectrum with peak assignments of Fmoc-lys-OAll in deuterated methanol.
Figure 6-10. 600 MHz $^1$H-NMR spectrum of protected lysines. Enlarged sections of the spectrum are compared to other precursors in the vicinity of 5 ppm to highlight the OAll protecting group. (A) Fmoc-lys(Boc)-OH in d$_6$-acetone has no vinyl protons. (B) Fmoc-lys(Boc)-OAll in d$_3$-chloroform has 3 vinyl protons. (C) Fmoc-lys-OH in d$_4$-methanol has a very small quantity of vinyl protons. (D) Fmoc-lys-OAll in d$_4$-methanol has 3 vinyl protons.
Figure 6-11. 600 MHz $^1$H-NMR spectrum of protected lysines. Enlarged sections of the spectrum are compared to other precursors in the vicinity of 1 ppm to highlight the Boc protecting group. (A) Fmoc-lys(Boc)-OH in d$_6$-acetone has a $t$-butyl methyl singlet at 1.4 ppm. (B) Fmoc-lys(Boc)-OAll in d$_3$-chloroform has a $t$-butyl methyl singlet. (C) Fmoc-lys-OH in d$_4$-methanol does not have a $t$-butyl methyl singlet. (D) Fmoc-lys-OAll in d$_4$-methanol does not have a $t$-butyl methyl singlet.
6.4. Discussion

Proposed here is the use of a high affinity cyclic RGD peptide in a biomimetic surface modification system for the facilitation of endothelial cell attachment and proliferation. Kantlehner et al. has incorporated c(RGDfK) into a biomaterial with positive results [6.25]. The lower case “f” depicts D-phenylalanine. The use of a D-amino acid is of no surprise in this situation, because in a 5-amino acid lactam, one amino acid must be a D-amino acid to lessen steric hindrance and allow for a synthetically reasonable ring closure with respectable yields [6.20, 26]. The particular use of phenylalanine also contributes to hydrophobic interactions with the integrin [6.27]. In this chapter two methods for synthesis of c(RGDfX) are described for incorporation into our biomimetic surfactant polymers.

The synthesis of c(RGDfK) using the first synthetic method was unsatisfactory. The method did not include extra washing steps to remove all residual palladium catalyst, and too much time was allotted for the cyclization reaction. The result was a product with the wrong mass, 619 instead of 604.31. The second synthetic method was implemented to improve results. The allyl deprotection was performed with the aid of the peptide synthesizer as was done by Kates et al. and Delforge et al. [6.28, 29]. They also used a sodium diethylthiocarbamate solution to wash residual palladium catalyst from the resin. The dithiocarbamate out-competes with the newly formed carboxylate terminus to form a stronger complex with the metallic catalyst; thus, enabling it to be washed from the resin.
The shorter time for the cyclization reaction was the main reason for the reduction of the undesirable product with an m/z of 619. HATU is a very reactive compound, and there is a good possibility for side reaction especially over a 16 h time period. In Figure 6-2, the product of a 16 h cyclization reaction is not c(RGDfK) with a m/z of 604.31, but has an m/z of 619. When a 1 h cyclization is performed (Figure 6-3D), both peaks of 605 and 619 m/z are present. Analytical rp-HPLC (Figure 6-5A) reveals that the ratio of these two products is ~1:1. Even shorter times of exposure should entirely produce a product with only a 604.31 m/z. Using rp-HPLC, c(RGDfK) (m/z = 605) was able to be purified (Figure 6-5B), and the resulting mass spectrum show no trace of the undesirable product with an m/z of 619.

Another issue with the cyclization reaction is whether to perform the reaction in the solid phase as McCusker et al. did or in the solution phase as Gurrath et al. [6.19, 22]. The solid phase method involves easier purification with fewer steps, higher yields, and the opportunity for automated synthesis. Solution phase cyclization requires high-dilution conditions, more extensive purification, and lower yields. However, the solid-phase method requires that the C-terminal amino acid has an appropriate side chain functional group for resin attachment. All amino acids that fit that requirement are chiral, and the cyclization reaction can result in racemization of the C-terminal α-carbon. The solution phase cyclization can be performed on a linear peptide with a C-terminal glycine, an amino acid with no side chain and no chiral α-carbon; therefore there is no chance for racemization.

With a few modifications to c(RGDfK), the cyclic RGD motif can be incorporated into our surfactant polymers for biomaterial surface modification. Haubner
et al. showed that the any amino acid can be put in the X position of c(RGDfX) with no affect on the peptide’s biological activity [6.21]. Thumshrin et al. used a c(RGDfE) peptide to bind to an αvβ3 integrin [6.30]. One of our peptide designs uses the c(RGDfE) motif, and attached to the side chain of glutamic acid is the triple serine spacer used in all the previous chapters to elevate the functional peptide above the biomaterial surface. At the end of the serine spacer is a lysine amino acid (Scheme 6-2). The successful synthesis of this peptide is shown in Figure 6-7. Lysine’s ε-amino is the only amine in the peptide, which allows for selective functionalization. Glutaric dialdehyde can be conjugated to the ε-amino of lysine using reductive amination as was done in Chapter 3. The peptide would now have an aldehyde functional group, which would allow for selective conjugation to the PVAm backbone (also as done in Chapter 3), resulting in a surfactant polymer that exhibits a cyclic-RGD motif elevated above the material surface.

Fmoc-lys-OAll was synthesized from Fmoc-lys(Boc)-OH for use in a cyclic RGD peptide for incorporation into a surfactant polymer. First allyl bromide was used to protect the α-carboxylate. The resulting product, Fmoc-lys(Boc)-OAll, had a new HPLC retention time of 22.4 min, instead of 18.7 min for Fmoc-lys(Boc)-OH (Figure 6-8A), and also displayed new vinyl protons from 5-6 ppm in the 1H-NMR spectrum (Figure 6-10B). Next TFA was used to remove the t-butyloxycarbonyl (Boc) protecting group to produce Fmoc-lys-OAll. rp-HPLC purification produced pure Fmoc-lys-OAll with a unique HPLC retention time of 15.0 min (Figure 6-8A) and the lack of a t-butyl methyl singlet at 1.44 ppm in the 1H-NMR spectrum.

The cyclic RGD motif can also be integrated into our surfactant polymer design with a PEO spacer as in Chapter 4. Scheme 6-4 shows the synthesis of a c(RGDfK)
Scheme 6-4. c(RGDfK(PEO220)) synthesis.
peptide with a PEO spacer. First, 3,6,9-trioxaundecanedioic acid (in excess) is loaded onto an acid labile solid support resin like the 2-chlorotrityl resin. Then, the Fmoc-lys-OAll that was synthesized earlier is coupled to the exposed carboxylate. Next, solid phase synthetic methods can be used to couple Fmoc-D-phe-OH, Fmoc-Asp(OBzl)-OH, Fmoc-Gly-OH, and Fmoc-Arg(Pbf)-OH. The C- and N- termini are then deprotected using a palladium catalyst and piperidine, respectively, and cyclization is then completed using HATU. The cyclic peptide is then cleaved from the resin using acetic acid and trifluoroethanol in chloroform. The resulting cyclic peptide is protected in such a way that there is only one carboxylate for the selective coupling onto the poly(vinyl amine) backbone of the surfactant polymer. Once the c(RGDfK) is coupled to PVAm, the peptide can be deprotected with 85% TFA.

Affinity of the cyclic RGD peptides for the \( \alpha_v\beta_3 \) integrin can be further increased by adding a methyl group to the amide backbone. N\(^{\alpha}\)-methylation has been used previously to increase affinity of some cyclic RGD peptides to the \( \alpha_{IIb}\beta_3 \) integrin [6.31, 32]. The peptide c(RGDf-N(Me)V), where the methyl group is added to \( \alpha \)-amine of valine, showed the greatest increase in affinity, approximately a one order of magnitude improvement over c(RGDfV) [6.33]. One drawback of this peptide is that valine does not provide a functionality for convenient bioconjugation, but Haubner \textit{et al.} showed that the \( 5^{\text{th}} \) amino acid’s side chain (in this case, the isopropyl group of valine) has little effect on integrin affinity [6.21]. Therefore, it is reasonable to conclude any peptide of the format c(RGDf-N(Me)X) would also show enhanced \( \alpha_v\beta_3 \) affinity. N-methyl amino acids can be either obtained commercially or prepared from Fmoc-protected amino acids by the method described by Freidinger \textit{et al.} [6.34]. In particular, the Fmoc -
MeGlu(OtBu)-OH amino acid is commercially available, which would allow for the synthesis of a c(RGDf-N(Me)E(SSSK)) peptide and also allow for easy incorporation into our surfactant polymers.

The cyclic RGD peptides in this chapter all bind with high affinity to the $\alpha_v\beta_3$; however, endothelial cells do contain other integrins that should not be ignored in creating a biomimetic surface. Some integrins, such as the $\alpha_5\beta_1$ integrin, are more responsible for establishing tight attachments with the surface [6.35-38], whereas, other integrins, such as the $\alpha_v\beta_3$ integrin, play a larger role in cell migration, angiogenesis, wound healing, and tumor metastasis [6.39-41], possibly due to its looser grip on its ligand/surface. There do exist cyclic RGD peptides for binding to $\alpha_5\beta_1$ integrins [6.42-44]. Also, antibody inhibition experiments by Mardilovich et al. showed that cell binding to a linear RGD peptide was mainly through the $\alpha_v\beta_3$ integrin; however, when the same linear RGD peptide was conjugated to the PHSRN synergy peptide separated by a spacer, cell binding was mainly through the $\alpha_5\beta_1$ integrin [6.45]. Therefore, it may be possible to conjugate the PHSRN synergy peptide to c(RGDfX) separated by a spacer to encourage binding through the $\alpha_v\beta_3$ integrin instead of through the $\alpha_v\beta_3$ integrin. Another improvement might be to also make the PHSRN peptide cyclic since it exists in the fibronectin crystal structure as another tight loop.

Antibody inhibition experiments by Mardilovich et al. showed that cell binding to fibronectin surfaces was more effectively inhibited by an anti-$\beta_1$ antibody then by an anti-$\alpha_5\beta_1$ antibody, which implies that an addition cell integrin containing the $\beta_1$ subunit also contributes to cell attachment [6.45]. It is possible that the $\alpha_4\beta_1$ integrin is responsible for this contribution. Peptides targeted for the $\alpha_4\beta_1$ integrin could also be incorporated into
our surfactant polymers to allow for a more complete biomimicry of fibronectin and a more complete cell attachment. Cardarelli et al. show that the cyclic Ada-C*GRGDSPC* peptide can block leukocyte adhesion with an IC$_{50}$ in the $\mu$M range [6.46]. Jackson et al. describe disulfide tetramer peptides and peptidomimetics that bind in the nM range [6.47]. Lin et al. describe a peptidomimetic based on the LDV peptide sequence that tightly binds to $\alpha_4\beta_1$ [6.48].

Specificity is another important consideration in designing nonthrombogenic biomaterial surfaces for endothelialization. Simple attachment of linear RGD peptides to a surface will not only enable attachment of endothelial cells, but also allow attachment of thrombogenic platelets mediated by the $\alpha_{I\beta 3}$ integrin. The c(RGDfX) peptides have three orders of magnitude greater affinity for the $\alpha_v\beta_3$ integrin compared to the $\alpha_{I\beta 3}$ integrin [6.21]. Koivunen et al. used phage display libraries to find disulfide RGD peptides that bind $\alpha_5\beta_1$ integrins and not $\alpha_{I\beta 3}$ integrins [6.42, 43]. Another possibility for ligands that are $\alpha_5\beta_1$ specific may lie within the bacterial integrin-binding protein, invasion, which binds to $\alpha_5\beta_1$ two orders of magnitude greater the fibronectin [6.49]. Finally, the peptidomimetic from Lin et al. shows 5 orders of magnitude greater affinity for the $\alpha_4\beta_1$ integrin over the $\alpha_{I\beta 3}$ integrin [6.48].

6.5. Conclusion

It is hypothesized that a material whose surface possesses the RGD peptide constrained in the proper conformation similar to the conformation of the RGD sequence
in fibronectin would support stronger attachment of endothelial cells, when compared to a surface with linear RGD peptides, thus better promoting surface endothelialization.

The c(RGDfX) peptides have a constrained conformation of the RGD sequence and should provide the desired effect of enhanced cell attachment. This study provides a proof of concept for synthesizing cyclic RGD peptides and provides a plan for their incorporation into our surfactant polymers. Surfactant polymers with cyclic RGD peptides could be used to produce nonthrombogenic, endothelialized cardiovascular biomaterials.

6.6. Acknowledgments

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6.7. References


Chapter 7. OTHER DESIGNS AND SYNTHESSES FOR SURFACTANT POLYMERS

7.1. FITC-PVAm(Dex:Hex)


The following experimental method was devised to show that, when exposed to whole blood, surfactant polymers do not desorb nor do they exchange with other surface active elements in whole blood. Fluorescently-labeled PVAm(Dex:Hex) [1:5] was prepared for total internal reflectance fluorescence (TIRF) experiments. The fluorescent label chosen was F6218, an acyl azide derivative of fluorescein (Scheme 7-1), which was obtained from Invitrogen (Carlsbad, California). At a ratio which corresponds roughly to a 1:5 F6218:dextran ratio, PVAm(Dex:Hex) [1:5] (15.5mg) and F6218 (0.18mg) were dissolved in 2 mL of anhydrous DMF (freshly distilled). The reaction vessel was sealed to prevent water vapor from interfering with the reaction. The mixture was allowed to react with constant stirring at 80°C for four hours. Upon heating to 80°C, the acyl azide rearranges to form an isocyanate, which readily reacts with hydroxyl groups in solution (Scheme 7-1). At this point, the newly formed conjugate is not yet fluorescent, and requires the fluorescein’s acetate groups to be deprotected. A 2 x 10⁻² M stock solution of potassium hydroxide in water was prepared. The basic stock solution was added to the reaction mixture to make the pH just above 9. After two hours of stirring at room
Scheme 7.1. F6218 acyl azide coupling to a carbohydrate (R-OH).
temperature, the reaction mixture, now fluorescently active, was brought to a more neutral pH by adding sodium bicarbonate in order to prepare the solution for dialysis. The reaction mixture was then dialyzed in pure water using a 3000 MWCO regenerated cellulose membrane. No fluorescent species were visually detected on the outside of the dialysis membrane, suggesting a near 100% completion of the conjugation reaction. After 24 hours, the solution was lyophilized to yield an orange product. For the TIRF experiment, the product, PVAm(fDex:Dex:Hex)[0.2:0.8:5] was dissolved in water at 2 mg/mL for the in-situ adsorption onto an OTS-modified quartz slide. (The OTS-modified quartz slide was prepared as in Section 4.2.4.1.) Inside the TIRF flow cell, fluorescence intensity was measured before and after exposure to whole blood. The fluorescence intensity did not decrease after exposure to whole blood indicating that highly surface active lipids within whole blood did not compete for binding to the surface, and those lipids did not exchange with the dextran surfactant polymer.

### 7.2. Fluorescently Labeled Peptides

Fluorescently labeled peptides were synthesized for use in solution-phase experiments for the quantification of ligand-receptor binding affinity. A succinimimidyl ester derivative of fluorescein (F-6130, Invitrogen, Carlsbad, California) was coupled to the N-terminus of disulfide, cyclic CRRETAWAC, and c(CRRETAWAC). F-6130 (2.66 μmol), c(CRRETAWAC) (2.66 μmol), and diisopropylethylamine (DIPEA) (10.6 μmol) were added to DMSO (1 mL) and allowed to stir at rt for 4 h. The reaction mixture was
purified by dialysis (regenerated cellulose membrane, 500 MWCO) for 24 h against water. The sample was lyophilized. A MALDI-TOF mass spectrum was acquired (Figure 7-1). Two main peaks were observed at 1093 and 1570, which were assigned to c(CRRETAWAC) (1092.5 calculated mass) and fluorescein-c(CRRETAWAC) (1567.97 calculated mass), respectively.

For other experiments, a succinimidyl ester derivative of fluorescein (F-6130, Invitrogen, Carlsbad, California) was coupled to the N-terminus of the GSSSGRGDSP peptide. F-6130 (2.95 μmol), c(CRRETAWAC) (2.95 μmol), and diisopropylethylamine (DIPEA) (11.5 μmol) were added to DMSO (1.2 mL) and allowed to stir at rt for 4 h. The reaction mixture was purified by dialysis against water (regenerated cellulose membrane, 500 MWCO) for 24 h and then lyophilized. A MALDI-TOF mass spectrum was acquired of the final product (Figure 7-2). Three main peaks were observed at 1382, 1454 and 1476, which were assigned to fluorescein-GSSSGRGDSP (desired product less an alanine), fluorescein-GSSSGRGDSP (1453.5 calculated mass), and fluorescein-GSSSGRGDSP (desired product with sodium ion), respectively. Notice that no peak was observed for the unlabeled peptide at 976, which would have corresponded to the peptide with no fluorescein.

Finally, a succinimidyl ester derivative of fluorescein (F-6130, Invitrogen, Carlsbad, California) was coupled to the lysine ε-amine of the c(RGDfK) peptide. F-6130 (2.95 μmol), c(RGDfK) (2.95 μmol), and diisopropylethylamine (DIPEA) (11.5 μmol) were added to DMSO (1.2 mL) and allowed to stir at rt for 4 h. The reaction mixture was purified by dialysis against water (regenerated cellulose membrane, 500 MWCO) for 24 h and then lyophilized. A MALDI-TOF mass spectrum was acquired
Figure 7-1. MALDI-TOF mass spectrum of fluorescein-c(CRRETAWAC). The peak at 1093 m/z was assigned to c(CRRETAWAC) (1092.50 calculated mass). The peak observed at 1570 was assigned to fluorescein-c(CRRETAWAC) (1567.97 calculated mass).
Figure 7-2. MALDI-TOF mass spectrum of fluorescein-GSSSGRGDSPA. Three main peaks were observed at 1382, 1454 and 1476, which were assigned to fluorescein-GSSSGRGDSP (desired product less an alanine), fluorescein-GSSSGRGDSP (1453.5 calculated mass), and fluorescein-GSSSGRGDSP (desired product with sodium ion), respectively. Notice that no peak was observed for GSSSGRGDSP (976 calculated mass).
(Figure 7-3). Three peaks in the spectrum; 1081, 1095, and 1117; were assigned to c(RGDfK(fluorescein)) (desired product with 1081.2 calculated mass), undesired product with mass 14 greater than desired product, and undesired product with sodium ion, respectively. No peak was observed for unlabeled c(RGDfK) peptide (604 or 619 calculated mass). The undesired product with mass 14 greater than desired product was discussed in the previous chapter (Sections 6.2.2, 6.3.1.2, 6.3.1.4, 6.3.2.2, and 6.4), where steps are outlined and tested for the elimination of that undesired product.

7.3. HBP3 Synthesis

Another heparin-binding peptide, GSSSGSPRRARVTDATETTITISWRTKT (HBP3), was synthesized, and methods are outlined for its incorporation into a surfactant polymer. Ingham et al. showed a peptide containing the HBP2 active sequence bound to heparin with a $K_d$ of 41 $\mu$M [7.2], and the extended native 22-residue peptide sequence, which is contained within HBP3, bound with a $K_d$ of 0.3 $\mu$M [7.3]. The arginines in the HBP3 sequence have been shown to be crucial for FN-III$_{13}$ binding to heparin as mutations at these locations obliterated binding in 0.15 M NaCl. Mutation at the lysine’s position also significantly decreased binding of HPB3 to FN-III$_{13}$ [7.4]. HBP3 was first synthesized to examine the feasibility on making a large peptide (29 amino acids) on a Knorr resin using standard solid-phase synthetic methods. The mass spectrum of this peptide is shown in Figure 7-4. The measured mass of 3340 is close to the calculated value of 3341.9 for the Fmoc-protected HBP3.
Figure 7-3. MALDI-TOF mass spectrum of c(RGDfK(fluorescein)). Three peaks in the spectrum; 1081, 1095, and 1117; were assigned to c(RGDfK(fluorescein)) (desired product with 1081.2 calculated mass), undesired product with mass 14 greater than desired product, and undesired product with sodium ion, respectively. No peak was observed for unlabeled c(RGDfK) peptide.
Figure 7-4. MALDI-TOF mass spectrum of Fmoc-HBP3. The observed product with a peak at 3340 m/z is the desired product (3341.7 calculated).
The incorporation of HBP3 into a surfactant polymer should result in higher affinity binding to EC HSPG, and the synthesis of this surfactant polymer is outlined in Scheme 7-2. First the entire 29 residue sequence is synthesized on a Knorr-2-chlorotrityl resin using standard solid phase peptides synthetic methods (Scheme 7-2A). Next, under mildly acidic conditions, the fully protected peptide is cleaved from the resin with the Knorr still attached to the C-terminus (Scheme 7-2B). Then, the C-terminal carboxylate is blocked by reacting it with excess tris(hydroxymethyl)aminomethane using EDC and NHS (Scheme 7-2C). Now, the N-terminus can be reacted with excess PEO diacid (Scheme 7-2D). Finally, the protected peptide along with the alkyl legs can be reacted onto the PVAm backbone, and then the peptide can be deprotected using 85% TFA (Scheme 7-2E).

7.4. Glycopeptide

A peptide was designed that would mimic a natural proteoglycan. A proteoglycan is a biological molecule that consists of a peptide backbone with sugar chains grafted onto the amino acid side chains. *In vivo*, sugars are added to a protein post-translation and are usually either: (1) O-linked to either a serine or threonine, and sometimes to a hydroxylysine; or (2) N-linked to an asparagine. However, in a laboratory, it is synthetically easier to couple a sugar to an amine such as the ε-amine of lysine’s side chain. Some Fmoc-protected non-natural amino acids are available for purchasing, in particular, 1,2 diaminopropanoic acid (Dap). This amino acid was chosen for linking to
Scheme 7-2. HBP3 surfactant polymer synthesis. Black blocks are TFA-labile side chain protecting groups. The red “L” block is the acetic acid labile resin linker.
Scheme 7-2 (cont.). HBP3 surfactant poolymer synthesis.
sugars because its chemical structure resembles serine with a β-amine instead of a β-hydroxyl. (Another option would be to use aspartic acid, whereupon coupling a sugar with an amine group, such as N-acetyl glucosamine, would result in an N-linked sugar to an asparagine exactly the same as found in cellular proteins). The first design iteration was a peptide with the following sequence: Mal-G-Dap(Mal)-G-Dap(Mal)-G-Dap(Mal)-G-Dap(Mal)-G, where Mal is Maltose. Molecular modeling of this glycopeptide revealed that in an extended conformation like a β-strand structure this glycopeptide would have all of its sugars on the same side of the peptide backbone (Figure 7-5A). However, this structure does not provide the desired bottle brush structure as found naturally in proteoglycans. A bottle brush glycopeptide would be well hydrated on all sides and would therefore resist protein adsorption when in contact with blood. The structure shown in Figure 7-5A is possibly amphiphilic and might associate with blood proteins.

To address this potential problem, another structure was designed (Figure 7-5B): Mal-G-Dap(Mal)-GG-Dap(Mal)-GG-Dap(Mal)-G. This glycopeptide, when in an extended conformation, places the maltose sugars on opposite sides of the peptide backbone. The extra glycine amino acids were added to the design in order to minimize steric issues that may be encountered during the grafting of the maltose sugars. The synthesis of this peptide is outlined in Scheme 7-3. The peptide, synthesized on a Wang resin, has its methyl trityl amine side chains deprotected using TFA (Scheme 7-3A). Maltose lactone is reacted with the amine side chains and terminus (Scheme 7-3B). The glycopeptide is cleaved from the Wang resin (Scheme 7-3C) and has a carboxylate C-terminus for conjugation onto poly(vinyl amine). This scheme most likely would work best for glycopeptides with small oligosaccharides.
Figure 7-5. Extended conformations of two glycopeptide designs: (A) Mal-G-Dap(Mal)-G-Dap(Mal)-G-Dap(Mal)-G-Dap(Mal)-G-Dap(Mal)-G and (B) Mal-G-Dap(Mal)-GG-Dap(Mal)-GG-Dap(Mal)-G-G. (G=glycine, Dap=diaminopropionic acid, and Mal=Maltose) The peptide backbones are green and the sugars are colored light blue.
Scheme 7-3. Glycopeptide synthesis.
Grafting large oligosaccharides with a high efficiency would probably prove difficult due to steric interference with neighboring saccharide chains. A more successful strategy would be to “grow” the oligosaccharide chains on the peptide using solid phase synthetic methods (Scheme 7-4). Fmoc-Dap(Alloc)-OH could be prepared by methods analogous to the preparation of Fmoc-lys(Alloc)-OH [7.5]. A peptide chain of multiple allyl-protected diaminopropionic acids could be prepared using Fmoc-chemistry and standard solid phase peptide synthetic methods. Next, the allyl and Fmoc protecting groups are removed with Pd(PPh₃)₄ and piperidine, respectively (Scheme 7-4A). An Fmoc-protected sugar amino acid could be prepared by the methods of Locardi et al. [7.6, 7], and then by successive coupling and deprotection cycles, oligosaccharides are “grown” on the peptide chain (Scheme 7-4B). Finally, the proteoglycan mimic is cleaved from the 2-Chlorotrityl solid support resin under mildly acidic conditions (Scheme 7-4C).

7.5. Future Surfactant Polymer Design

As it has been discussed throughout this work, there are several different ways in which EC (endothelial cells) attach with the ECM (extracellular matrix). The αvβ3 integrin plays an important role in cell migration and wound healing; the α5β1 integrin is important for forming strong attachments, and the HSPG (heparan sulfate proteoglycans) are important for forming initial surface attachments. In Chapter 4, it was shown that additional benefit can be achieved by incorporating more than one cell binding motif into a surfactant polymer. Figure 7-6 shows a future design of a surfactant polymer that
Scheme 7-4. Solid-phase glycopeptide synthesis.
Figure 7-6. Design of a biomimetic surfactant polymer that aims to take advantage of all the ligand – EC receptor interactions discussed within this thesis. Attached to a PVAm backbone (A) are hydrophobic legs (B) for adsorption onto the material surface, small oligosaccharides (C) for the prevention of nonspecific protein adsorption, high-affinity cyclic peptides (D) for binding to EC αvβ3 integrins, a high affinity cyclic peptide (E) for binding to EC α5β1 integrins connected to a cyclic PHSRN synergy peptide (F) to increase α5β1 interaction by a PEO tether (gold), and heparin-binding peptides (G) for binding with EC HSPG.
incorporates all cell binding motifs discussed in this work. The surfactant polymer contains small oligosaccharides for the prevention of nonspecific protein adsorption, high-affinity cyclic peptides for binding to EC \( \alpha_5\beta_3 \) integrins, a high affinity cyclic peptide for binding to EC \( \alpha_5\beta_1 \) integrins connected to a cyclic PHSRN synergy peptide to increase \( \alpha_5\beta_1 \) interaction by a PEO (poly(ethylene oxide)) tether, and heparin-binding peptides for binding with EC HSPG.

The rationale for this design is that throughout the biochemical literature surveyed in this dissertation and observations made from our own experiments [7.8], it is obvious that ECs require and benefit from multiple means of anchorage. At first glance this surfactant polymer may seem complicated; however, each individual peptide ligand in this polymer can be synthesized in parallel using synthetic methods outlined throughout this dissertation. Then, the individual peptide ligands can be coupled to the poly(vinyl amine) (PVAm) backbone towards the end of the surfactant polymer synthesis. The relative ratio of the peptide ligands in the surfactant polymer can be determined using solution \(^1\)H-NMR and can be modulated by varying the relative mole fraction of each individual peptide ligand during the step of coupling to the PVAm backbone.

Another possibility for introducing multiple EC anchorage functionalities onto a biomaterial surface would be to mix different surfactant polymers, each containing a single different EC anchorage ligand, in solution. Then, the blend of surfactant polymers would adsorb onto the surface. One possible drawback of this method is that one particular surfactant polymer could have a slightly higher surface activity than the other surfactant polymers in solution. Since the number of sites for adsorption onto the surface are very limited compared to the large number of surfactant polymer molecules in
solution, then the modified surface could result in having only one kind of surfactant polymer adsorbed. Another possibility is that each kind surfactant polymer will preferentially adsorb with surfactant polymers of its own kind, resulting in a surface consisting of large patches with each patch containing only a single kind of EC anchorage ligand. The use of an aqueous blend of surfactant polymers to create a surface with multiple ligands would then require experiments to quantify each kind of EC anchorage ligand on the surface and experiments to measure the distribution of each kind of EC anchorage ligand on the surface.

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7.7. References


Chapter 8. BIBLIOGRAPHY


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containing peptide ligands from the heparin binding domain of fibronectin."


Sagnella, S. M., F. Kligman, E. H. Anderson, J. E. King, G. Murugesan, R. E. Marchant


Unpublished result, A MALDI-TOF mass spectrum was acquired for a newly cleaved peptide, showing equal intensity for the two species: [M + H]+ and [M + Pbf + H]+. However, separation of the two fractions and integration of their peaks at both 220 nm and 280 nm using analytical scale rp-HPLC showed that the actual relative concentration was closer to 90:10. Therefore, MALDI-TOF over exaggerates species with Pbf groups.