PEPTIDE MODIFIED PDMS FOR IMPROVED VASCULAR CELL INTERACTIONS

PEPTIDE MODIFIED PDMS: SURFACE MODIFICATION FOR IMPROVED VASCULAR CELL INTERACTIONS

By

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ABSTRACT

Many of the materials used today for cardiovascular implants exhibit good bulk mechanical properties but fail to provide desirable surface properties for reducing thrombogenicity and promoting tissue integration. In fact, biological responses at the blood-material interface, including non-specific protein adsorption, coagulation, and platelet adhesion and activation significantly limit the use of currently available materials in many blood contacting applications. As our understanding of the biological responses to foreign materials has grown, so too has the potential for creating 'bioactive' materials capable of inducing and directing beneficial cellular processes. One promising technique for circumventing undesirable blood-biomaterial interactions involves seeding vascular endothelial cells (ECs) onto synthetic vascular grafts as a means of exploiting the physiological anticoagulant characteristics of the endothelium. Methods for improving cell retention on these constructs include immobilization of cell recognition motifs on the biomaterial surface in order to improve interactions between cells and the synthetic substrate. However, there remains the need to better understand the interactions between surface bound ligands and cells, and the role of linker molecule chemistry on ligand bioactivity and cellular response. In the current work, a novel method was optimized for modifying poly (dimethylsiloxane) (PDMS) with cell adhesion peptides tethered via a heterobifunctional allyl-, NSC-terminated polyethylene oxide (PEO) linker molecule. These novel surfaces combine the protein repellant property of PEO with the cell binding property of cell adhesion peptides. It was found that surfaces modified in this manner

reduced protein adsorption to PDMS while increasing cell adhesion. Therefore the use of a generic PEO linker molecule was shown to be a very promising method of reducing non-specific protein interactions while maintaining ligand bioactivity.

Silicone surfaces were also modified with diaminobutane (DAB) dendrimers in an attempt to increase the surface capacity for attachment of biomolecules and to compare the effect of surface peptide density with ligand mobility. Grafting cell adhesion peptides via surface bound dendrimers was found to increase the surface peptide density when compared to peptides grafted via the PEO spacer alone. However, cell adhesion was not significantly improved on the dendrimer-peptide modified surfaces compared to PDMS controls. This observation provides evidence that the properties of the linker molecule used for attachment of cell adhesion peptides to a biomaterial surface may be a critical factor in determining peptide bioactivity. In this case the peptides bound to the surface via the highly mobile linear PEO linker showed increased cell adhesion when compared to peptides linked via the rigid, highly branched dendrimer. It is therefore hypothesized that ligand mobility on a biomaterial surface may significantly influence ligand-cell receptor interactions to an even greater extent than surface peptide density.

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I would like to dedicate this work to my family for inspiring me to achieve great things in all aspects of life.

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LIST OF ABBREVIATIONS

APC	activated protein C
ATIII	antithrombin III
EC	endothelial cell
ECM	extracellular matrix
ePTFE	expanded polytetrafluoroethylene
NO	nitric oxide
PAI-I	plasminogen-activator-inhibitor
PAMAM	polyamidoamine dendrimer
PDMS	poly(dimethylsiloxane)
PEO	polyethylene oxide
Pt	platinum
RGD	arginine-glycine-aspartic acid (Arg-Gly-Asp) peptide
SMC	smooth muscle cell
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
TF	tissue factor
t-PA	tissue plasminogen activator
TAT	thrombin–antithrombin
vWF	von Willebrand factor

1 INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of premature mortality in the Western World, accounting for 16.7 million deaths worldwide in 2003[1]. In Canada in 2002, greater than thirty percent of all deaths resulted from CVD[2] resulting in an associated economic cost for treatment of more than $18 billion[3]. According to the American Heart Association, in 2003, cardiovascular related illnesses led to the death of more than 900 000 Americans with an associated economic burden of US$368.4 billion [4]. Clearly there are both significant therapeutic and economic incentives for developing effective treatments for cardiovascular related illnesses.

Of the various cardiovascular related illnesses, the narrowing of the coronary arteries due to the deposition of plaque and consequent formation of atherosclerotic lesions, known as coronary artery disease (CAD), accounts for greater than half of all CVD deaths[2,4]. Current treatment consists of coronary angioplasty or peripheral artery bypass grafting using autologous vessels including the mammary artery and saphenous vein. However, the supply of healthy native vessels from one individual is often insufficient for multiple bypass or repeat procedures. Furthermore, autologous grafts are prone to failure due to a variety of adverse reactions associated with the graft procedure. Therefore, other sources for arterial replacements, including synthetic, natural, and bio-hybrid conduits, are in great demand.

A biomaterial is defined as a non-viable material used in a medical device intended to interact with biological systems[5]. Over the last half century, biomaterials have been used in a variety of clinical applications for which 'biocompatible' materials,

defined as materials that elicit an appropriate host response for a given application, are required. In blood contacting applications, a material may be referred to as biocompatible, or "blood compatible", if it is capable of functioning in contact with blood without inducing reactions which negatively affect the performance of the device. Early biomaterials, including metals, ceramics, and polymers, were selected due to their ability to elicit a minimal response from host tissues, and were therefore termed "bioinert". To address the need for vascular graft alternatives, synthetic vascular grafts made from relatively bioinert materials such as polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTFE) have been successfully developed for clinical use in large diameter (>5 mm) arterial reconstructions[6]. However, biological responses at the blood-material interface remain a significant limiting factor in the development of small diameter (<5 mm) vascular grafts.

It is widely accepted that the initial event that occurs when a polymeric material comes in contact with blood is the adsorption of plasma proteins. All subsequent interactions between the biological environment and the implant are therefore mediated by the proteins on the material surface. The result is an assortment of reactions including activation of the coagulation pathway, the adhesion and activation of platelets, and the initiation of the complement cascade[7]. Consequently, eliminating or controlling the adsorption of proteins on a biomaterial surface following exposure to blood is highly desirable. In order to achieve this goal, non-fouling organic polymers may be grafted to the biomaterial surface. One polymer which has shown considerable ability to reduce protein adsorption in this manner is polyethylene oxide (PEO)[8-11]. However, though

surface grafting of PEO on the luminal surface of small diameter vascular prosthetics extends graft patency by significantly reducing plasma protein adsorption, this technique has yet been shown to eliminate protein adsorption and thrombus formation in its entirety[12,13]. While a variety of other techniques have been used to reduce the thrombogenicity of biomaterials intended for small diameter vascular graft applications, none have yet resulted in long term graft patency *in vivo*[14-16].

As our understanding of biological responses to foreign materials has grown, so too has the potential for creating 'bioactive' materials capable of inducing and directing beneficial cellular processes *in vivo*. A variety of bioactive materials have been developed which incorporate highly specific biological recognition molecules with the goal of preventing coagulation, increasing fibrinolytic activity, or encouraging endothelial cell adhesion and proliferation[17]. For example, extensive work has been performed on immobilizing heparin to biomaterial surfaces in order to impart anticoagulant surface properties[18]. Clot-dissolving surfaces have also been generated by covalent attachment of lysine and subsequent binding of plasminogen to polyurethane surfaces resulting in successful induction of fibrinolytic activity[19]. Although initial results are promising, neither of these examples have yet to lead to clinically useful vascular prostheses. Another promising technique for circumventing undesirable blood-biomaterial interactions involves seeding vascular endothelial cells (ECs) onto synthetic vascular grafts as a means of exploiting the physiological anticoagulant characteristics of the endothelium[20-22]. At present, the success of EC seeded vascular grafts has been limited largely due to poor EC retention upon exposure to blood flow[23]. Methods for

improving cell retention include immobilization of cell recognition motifs on the biomaterial surface in order to improve interactions between cells and the synthetic substrate. Manipulation of cell integrin interactions with bioactive extracellular matrix (ECM) proteins covalently grafted on synthetic biomaterials has been shown to be promising for inducing and directing various desirable cellular processes. For example, both cell adhesion peptides[25, 30, 111] and growth factors[26-28] have been incorporated into materials to optimize cell adhesion and proliferation. However, there is growing evidence that bioactive ligand mobility is an important factor for effective interaction with cell integrins in order to elicit maximum cell signal transduction and response[29,30,140]. Thus, rather than being immobilized directly to a synthetic surface, ligands may be attached to a substrate via flexible organic polymer tethers such as PEO in an attempt to improve ligand-receptor interactions between the polymer surface and cells[31].

Many of the materials used clinically for implanted device applications exhibit excellent bulk properties but fail to provide desirable surface properties for reducing thrombogenicity and promoting tissue integration. Poly(dimethylsiloxane) (PDMS) has found widespread use in biomedical applications over the past three decades as a result of its various advantageous chemical and physical properties[32]. However, silicone polymers are highly hydrophobic and readily adsorb biomolecules including proteins which mediate subsequent undesirable biological reactions and limit its use in blood contacting applications[33]. In the present work, surface passivating PEO was covalently grafted to PDMS. This acts as a highly mobile linker for the subsequent attachment of cell adhesion peptides. Two novel coupling chemistries for attachment of PEO to PDMS were

examined and characterized. Subsequent surface grafting of cell adhesion peptides was quantified by ^{125}I radiolabeling techniques. Techniques for increasing surface ligand binding capacity were also explored using dendrimer polymer chemistry. The effect of all surface modifications on cell adhesion peptide grafting and endothelial cell adhesion is reported.

2 LITERATURE REVIEW

2.1 Blood Vessel Anatomy and Physiology

Blood vessels, consisting of both arteries and veins, are the primary components of the cardiovascular system and are responsible for the transport of blood throughout the human body. Arteries consist of three anatomical layers: the intima, media, and adventitia (Figure 2.1). The intima consists of a thin lining of endothelial cells which comprise the endothelium and are bound to subendothelial connective tissue and the basement membrane. The media consists largely of vascular smooth muscle cells and is responsible for the vasocontrol of the artery. This layer varies greatly in thickness dependant on the location of the artery within the circulatory system. The outermost layer, known as the adventitia, is comprised mainly of connective tissue and nerves.

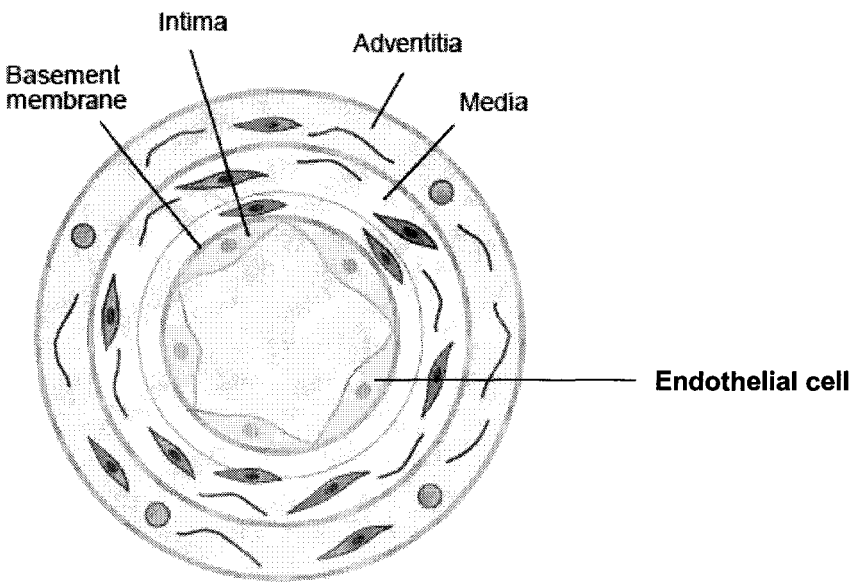


Figure 2.1. The anatomical structure of arteries, from [116].

2.1.1 The Vascular Endothelium

Vascular endothelial cells form the innermost lining of blood vessels and comprise the interface between blood and the extravascular space of surrounding tissues. These cells play a dynamic role in hemostasis, regulating both coagulation and fibrinolysis. Each EC provides an interactive site upon which a variety of receptor-mediated interactions may take place giving the endothelium the ability to respond to a wide range of environmental stimuli. These interactions initiate intracellular signaling pathways that result in precise hemostatic and fibrinolytic control. The unperturbed endothelium is non-thrombogenic and effectively separates flowing blood from the thrombogenic surrounding tissues.

Endothelial thromboresistance results from the production of a number of anti-platelet and anticoagulant substances. Thrombomodulin (TM), expressed on the surface of ECs, is a thrombin binding glycoprotein that results in a conformational change in thrombin. Thrombin bound to TM converts zymogen protein C to the anticoagulant enzyme activated protein C (APC). APC increases fibrinolytic activity through the inactivation of tissue plasminogen-activator-inhibitor (PAI-I), resulting in increased fibrin clot dissolution[39]. This process is collectively referred to as the protein C anticoagulant pathway. Thromboresistance is further imparted by heparan sulfate, a proteoglycan located on the EC surface which induces a conformational change in antithrombin III (ATIII), a glycoprotein, accelerating the inhibition of thrombin by ATIII[117]. In addition, tissue factor pathway inhibitor (TFPI), which is also expressed on the surface of ECs, complexes with factor Xa and acts to inhibit the tissue factor-factor

VIIa complex of the extrinsic coagulation pathway [117]. Fibrinolytic activity is further enhanced by the secretion of tissue plasminogen activator (t-PA). Finally, prostacyclin and nitric oxide (NO) act as vasodilators and inhibitors of platelet function.

ECs also possess the ability to assume a pro-coagulant state upon stimulation by vascular injury and a variety of cytokines. Perturbation of the endothelium results in the synthesis and secretion of von Willebrand factor (vWF) and PAI-1. Von Willebrand factor plays a critical role in the binding and transport of factor VIII to localized areas of vascular damage. vWF also binds to glycoprotein Ib and IIb/IIIa on platelet membranes and to exposed collagen, therefore promoting the adhesion of platelets at the site of vascular injury[118]. Tissue factor (TF), a crucial proponent of the extrinsic coagulation cascade, is produced by ECs and binds factor VIIa. The resulting complex converts factor X to factor Xa. The procoagulant activity of the endothelium results in the activation of platelets, and the formation of thrombin and fibrin clot.

2.2 Biomaterials for Cardiovascular Graft Applications

Coronary and peripheral vascular bypass grafting is now performed in more than one million cases annually in the United States and Europe[34]. Autologous vessels including the saphenous vein and mammary artery are the current vessels of choice for these procedures. However, the use of autologous vessels for bypass grafting is not without significant complications. For example, autologous grafts are prone to generation of thrombi, neointimal formation, atherosclerosis, aneurysm, spasm and contraction when transplanted into high pressure coronary circulation[35,36]. The principal polymeric graft

materials used in peripheral vascular reconstructions are woven polyethylene terephthalate (Dacron) and expanded polytetrafluoroethylene (ePTFE). However, due to the thrombogenicity of the internal surface of these grafts and the formation of intimal hyperplasia, their use in applications less than 5mm in diameter, such as coronary circulation, is limited.

Alternative materials for this application include both synthetic and natural polymers. Various synthetic materials, such as polyurethane, which has been used in ventricular assist devices (VAD) [37], have bulk physical characteristics that may be ideal for use in bypass procedures including similar compliance to native arteries [38]. However, synthetic materials have largely proven to be thrombogenic, limiting their applicability for generation of small diameter conduits[19]. Natural biomaterials, such as collagen, may be used to generate scaffolds for naturally derived biological or biohybrid grafts. Though this approach may present the possibility for highly biocompatible graft materials, they remain difficult to modify and more importantly have limited physical and chemical properties. Hence, the use of current biomaterials in blood contacting applications remains limited.

2.3 Coagulation and the Hemostatic Response

Blood coagulation is the naturally occurring hemostatic response which serves to arrest bleeding from damaged blood vessels. The process involves a complex set of interdependent reactions involving platelets and numerous plasma proteins resulting in the formation of a fibrin clot[39]. Inactive factors are enzymatically activated through a

cascade of reactions that follow two independent pathways of coagulation. The two pathways, known as the intrinsic and extrinsic coagulation pathways, are initiated by surface contact and exposure to damaged blood vessels respectively (Figure 2.2).

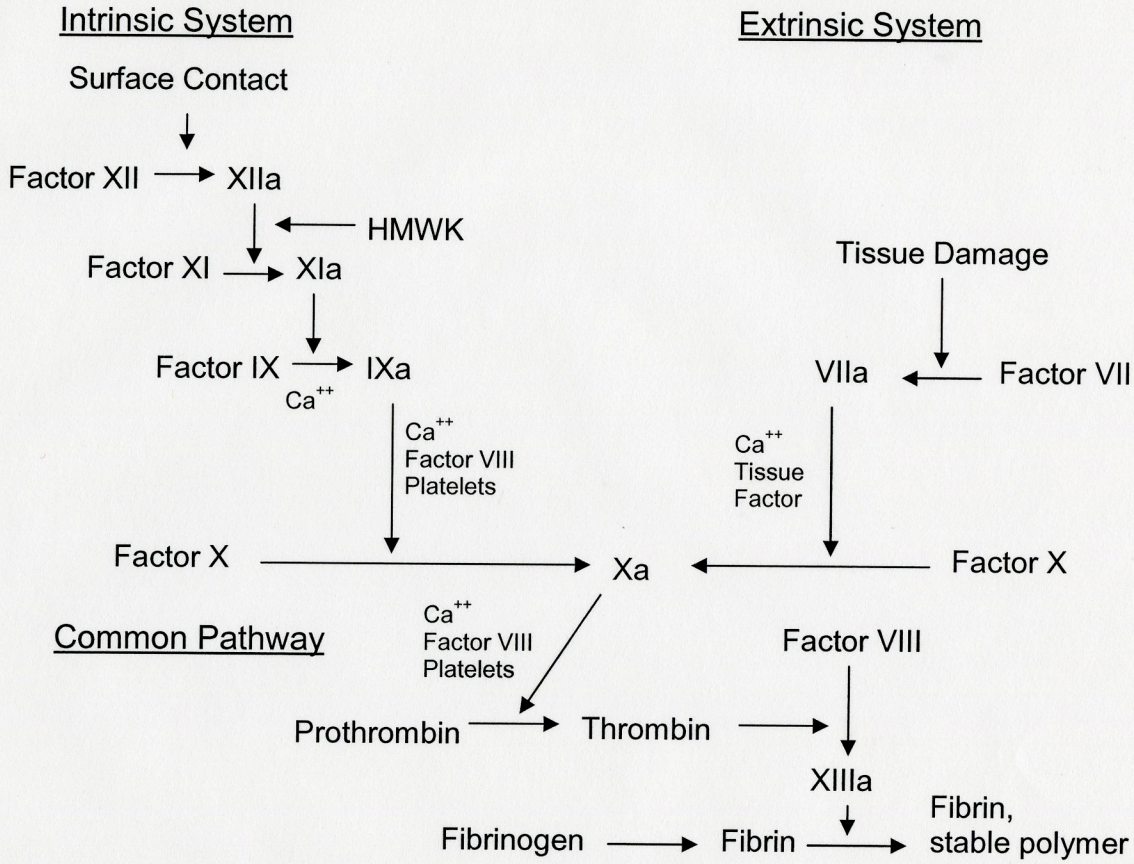


Figure 2.2 Schematic representation of blood coagulation pathways. Roman numerals represent enzyme or zymogen factors. Arrows indicate action of enzyme on zymogen or protein. HMWK refers to protein High Molecular Weight Kininogen, (from ref. 40).

The extrinsic coagulation pathway is initiated when blood is exposed to damage in the vascular wall or contact with extravascular tissues. This pathway is of particular importance following vascular trauma. The intrinsic pathway is initiated when blood is exposed to any foreign surface not commonly present in the intact vascular endothelium and is therefore of particular importance in the development of blood-contacting biomaterials. Upon activation by either pathway, inactive factors, or zymogens, enzymatically activate the next factor in the cascade ending with the conversion of prothrombin to thrombin. Thrombin activates platelets causing them to become adhesive and release factors which help to further amplify the cascade. Thrombin also catalyzes the polymerization of fibrinogen resulting in the formation of a fibrin clot. The coagulation cascade can be amplified by means of a variety of positive feedback mechanisms which result in the rapid formation of a clot.

When biomaterials are exposed to blood, a series of reactions occur which are generally referred to as the host response. These responses include coagulation, platelet adhesion and activation, complement activation and inflammation. These events are often detrimental to the success of cardiovascular implants and it is therefore desirable to generate materials capable of circumventing such responses.

2.4 Blood-Material Responses in the Presence of Biomaterials

2.4.1 Coagulation

As noted, the non-specific adsorption of proteins is the first event to occur in blood–material interactions[41]. This thermodynamically driven phenomenon is due in part to the high surface activity of amphipathic, high molecular weight proteins and the subsequent van der Waals, electrostatic, and entropic interactions with material surfaces[8,42]. Protein adsorption is followed by platelet adhesion, complement activation or inflammatory cell infiltration, and endothelial cell (EC) and smooth muscle cell (SMC) migration[7,43]. Upon exposure of a material to a protein rich solution such as blood, proteins present in high concentrations regardless of surface affinity are initially adsorbed. These proteins are then replaced over time by proteins present in lower concentrations but with higher surface affinity in a process commonly referred to as the Vroman effect[44]. All interactions with the surface that follow are dependent on the properties of the adsorbed protein layer including protein surface coverage, composition, and conformational state[45,46]. In fact, many of the non-specifically adsorbed proteins include those responsible for initiating coagulation, platelet activation, and activation of the complement system. Studies focused on protein adsorption on biomaterial surfaces from isolated protein solutions or plasma have shown that fibrinogen, involved in platelet adhesion[47,48] and the formation of fibrin, adsorbs rapidly to the surface and is replaced over time by HMWK[49]. FXII and FXIIa have also been observed on material surfaces further suggesting that materials that contact blood result in the stimulation of the intrinsic pathway of coagulation[50]. The intrinsic pathway of coagulation may also be

triggered by platelets, since platelets have been reported to be activated by direct binding to fibrinogen adsorbed on biomaterial surfaces and to promote cleavage of factor XI to factor XIa[51].

The extrinsic pathway of coagulation may also play a role in thrombin generation upon exposure of synthetic materials to whole blood as a result of TF expression by monocytes[52,53]. Hong et al.[54] found that thrombin–antithrombin (TAT) formation on polyvinyl chloride (PVC) was negligible in both plasma and platelet-rich plasma, while significant levels of TAT were observed upon exposure to whole blood containing leukocytes. However, the extrinsic pathway is more likely to play a role in the propagation of coagulation rather than in its initiation, as in vitro studies by Lindmark et al.[55] have shown that up-regulation of TF expression in monocytes is not immediate and may take up to 15 minutes. Therefore, it appears that both the intrinsic and extrinsic pathways may play a role in coagulation in the presence of biomaterials, though their relative importance requires further exploration.

2.4.2 Complement Activation

The complement system is an important component of the human body's defense mechanism against infection and foreign substances. This system consists of over 20 plasma proteins including both enzymes and binding proteins. Complement activation may occur via two pathways known as the classical and alternative pathways and coincide at a common terminal pathway (Figure 2.3). Complement activation is known to occur in the presence of biomaterials including catheters and synthetic vascular grafts[56].

Resulting increases in leukocyte adhesion and inflammation are a common cause of vascular graft failure[134,135]. The presence of a biomaterial is believed to activate the complement system via the alternative pathway[7]. It is believed that the extent of activation depends on the tendency of the biomaterial to bind Factor B rather than Factor H[57]. Binding of Factor H would lead to the inactivation of C3b resulting in the termination of the complement cascade (Figure 2.4). However, there is also evidence that the complement system may be activated by the classical pathway in the presence of biomaterials[58]. Evidence also appears to suggest that the coagulation cascade and the complement cascades are interactive. Therefore, the thrombogenicity of a biomaterial may play an important role in complement activation and in the extent of biomaterial induced inflammation. The exact process by which biomaterials activate the complement system and the extent to which each pathway contributes to this process remains a matter of contention.

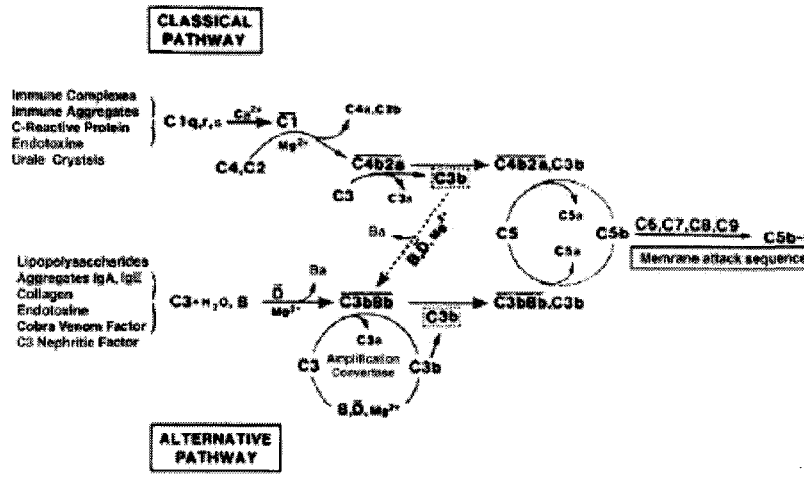


Figure 2.3. Pathway of complement activation [60]. Copyright JB Lippincott.

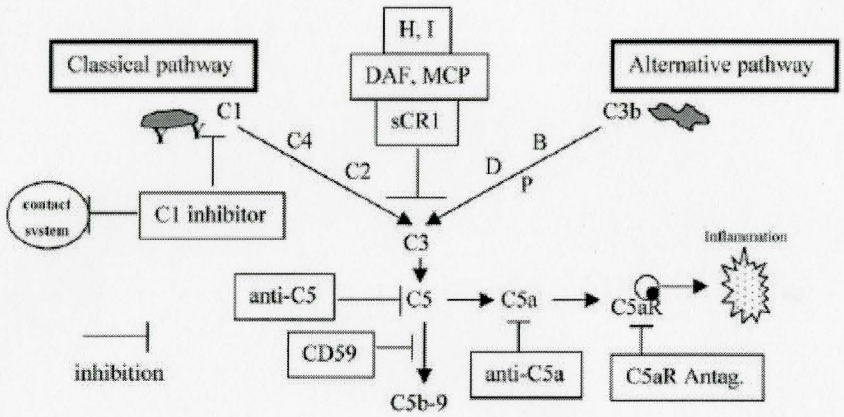


Figure 2.4. Pathway of complement inactivation [61]. Copyright Elsevier.

2.4.3 Platelet Adhesion/activation

Platelets are nonnucleated, disk-like components of blood which play a vital role in hemostasis by helping to generate a platelet plug upon vascular injury (Figure 2.5). Circulating platelets may become activated upon contact with various thrombogenic agonists including collagen exposed by injured endothelium, plasma proteins including thrombin and fibrinogen, inflammatory cells, and artificial surfaces. Platelet activation involves the interaction of an agonist to specific receptors on the platelet plasma membrane[62]. Activated platelets undergo several biological responses resulting in platelet adhesion and aggregation, and the release of various biochemical compounds including platelet factor 4, thrombospondin, β-thromboglobulin, ADP, thromboxane B_2, and serotonin[62]. In the presence of a biomaterial, platelets may adhere to the surface via interaction between the platelet surface receptor GPIIb/IIIa and the adsorbed proteins, particularly fibrinogen, on the biomaterial surface. However, platelet activation may also

result from non-adhesive encounters with a biomaterial resulting in downstream platelet aggregation and emboli formation. It is generally thought that biomaterial-induced platelet activation occurs as a result of the generation of thrombin due to activation of the coagulation cascade. However, the inhibition of thrombin and kallikrein has been used unsuccessfully to reduce platelet activation suggesting that the activation process may be mediated by agonists other than thrombin [63].

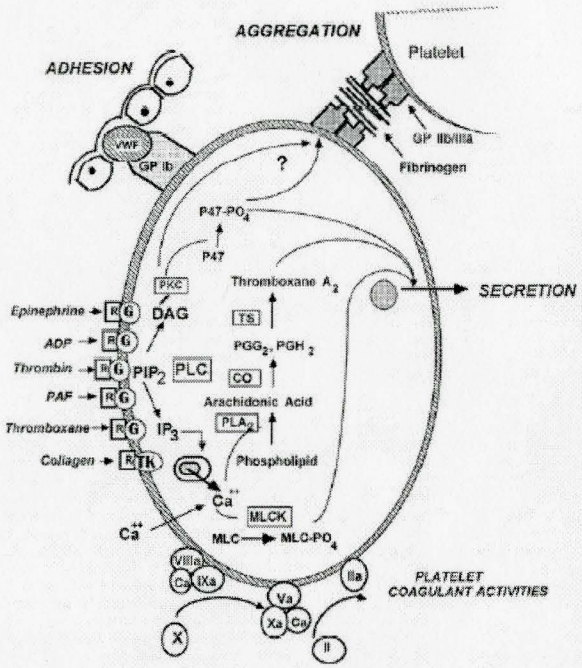


Figure 2.5. Schematic representation of normal platelet responses. Adapted from [64].

2.5 Poly (dimethylsiloxane) (PDMS) as a Biomaterial

Silicone elastomers, such as poly(dimethylsiloxane), have been widely used in medical applications in the past three decades as a result of their physiological inertness, low toxicity, and good thermal and oxidative stability[32]. In fact, PDMS was selected

along with low density polyethylene (LDPE) as one of the primary reference materials for validation of standardized and novel materials in assessment of biocompatibility in 1984[65]. The biocompatibility of PDMS with respect to complement activation, inflammation, and general tissue responses has been well investigated[65, 66]. Due to its good mechanical properties and good biocompatibility in various applications, PDMS in the form of gels, elastomers, and copolymers have been used to fabricate an assortment of medical devices including blood pumps, cardiac pacemakers, catheters, breast implants, orthopedic pads, oxygenators, contact lenses, finger joints, and drug delivery systems[67].

2.5.1 PDMS Chemistry

PDMS is a silicone with the basic structure shown in Figure 2.6. This polymer can be modified by replacing the methyl groups with a variety of other functional groups including vinyl, hydrogen, and silanol. These substitutions allow for curing or crosslinking, as well as reactions for surface modification of the polymer.

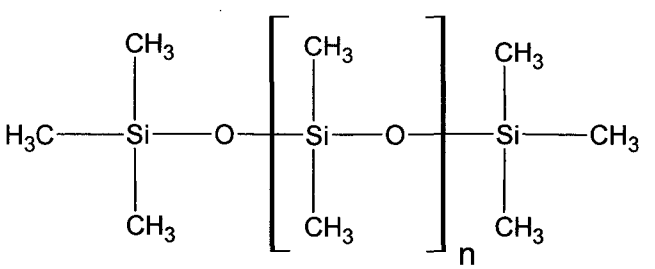


Figure 2.6. Chemical structure of poly(dimethylsiloxane).

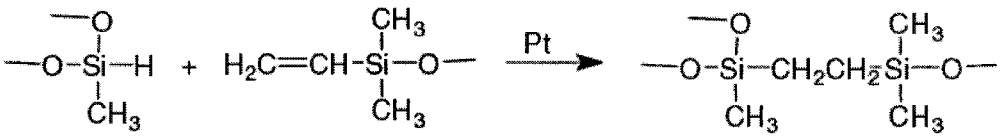
Various methods have been investigated for introducing reactive functional groups on the PDMS surface. Hydrosilylation involves the addition of Si-H functional groups to π-unsaturated, vinyl, or allyl groups. This reaction has been used as a method of introducing various R groups onto polysiloxane chains through a platinum catalyzed reaction of olefins with $(MeHSiO)_n$[68].

Due to the array of functional groups that may be substituted in the PDMS structure, a variety of curing methods are possible using various different prepolymer chemistries. The most common PDMS prepolymers, their curing processes, and resultant functionalized PDMS polymers are described below.

(1) Vinyl functional polymers.

Vinyl terminated polymers are commonly used in addition cure systems. The bond forming chemistry is a platinum catalyzed hydrosilylation reaction which proceeds according to the reaction scheme (2-1). The platinum (Pt) based catalyst system is usually dissolved in alcohol, xylene, divinyl siloxane or cyclic vinyl siloxanes. An advantage of this cure system is that no byproducts are formed.

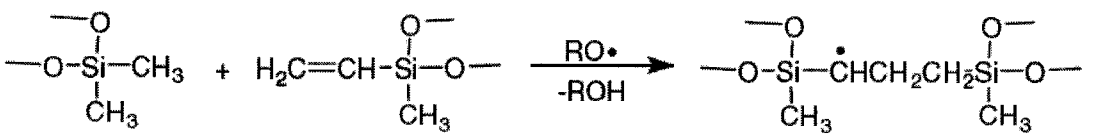
Scheme (2-1) (from [69]):



High temperature vulcanization (HTV) is used for generation of vinylmethylsiloxanes with the use of peroxide as activator. Commonly used peroxides include dibenzoyl

peroxide, dicumylperoxide, and bis(dichlorobenzoyl) peroxide. Free radical coupling of vinyl and methyl groups is usually initiated by peroxides at reaction temperatures of 140°-160° (scheme (2-2)).

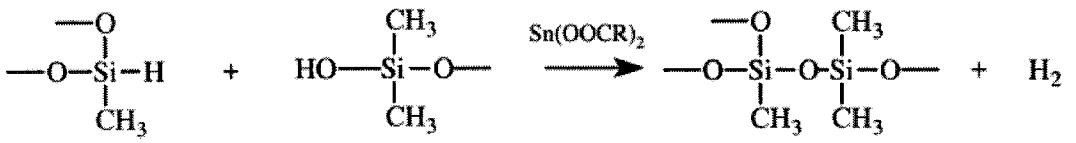
Scheme (2-2) (from ref. 69):



(2) Hydride functional polymers.

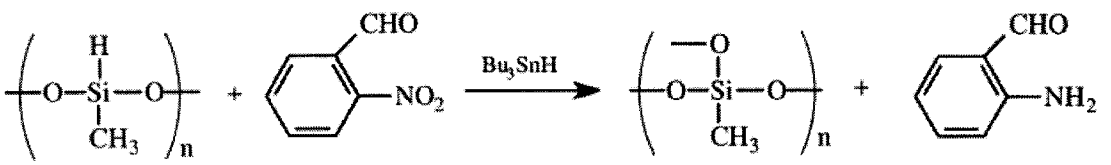
Hydride functional siloxanes are commonly used in three main classes of reactions: hydrosilylation (scheme (2-1)), dehydrogenative coupling (scheme (2-3)) and reduction (scheme (2-4)). Methylhydrosiloxane-dimethylsiloxane copolymers are commonly formed by addition cure techniques and have more controllable reactivity than homopolymers resulting in stronger polymers with lower cross-link density. When dehydrogenative coupling is employed, hydroxyl functional materials are reacted with hydride functional siloxanes in the presence of bis(2-ethylhexanoate)tin, zinc octoate, dibutyldilauryltin, iron octoate or other metal salt catalysts.

Scheme (2-3) (from ref. 69):



Chemoselective reduction is accomplished by proper choice of reaction conditions which result in hydride transfer (scheme (2-4)).

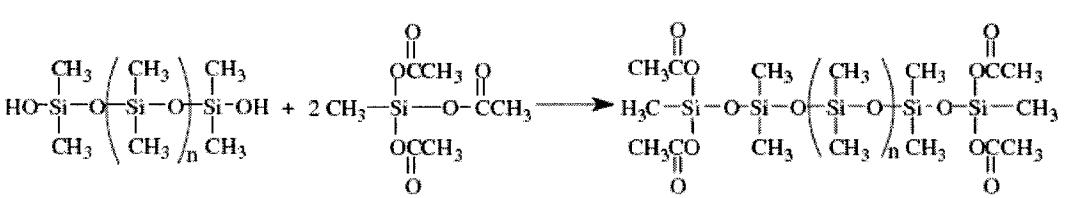
Scheme (2-4) (from ref. 69):



(3) Silanol Functional Polymers

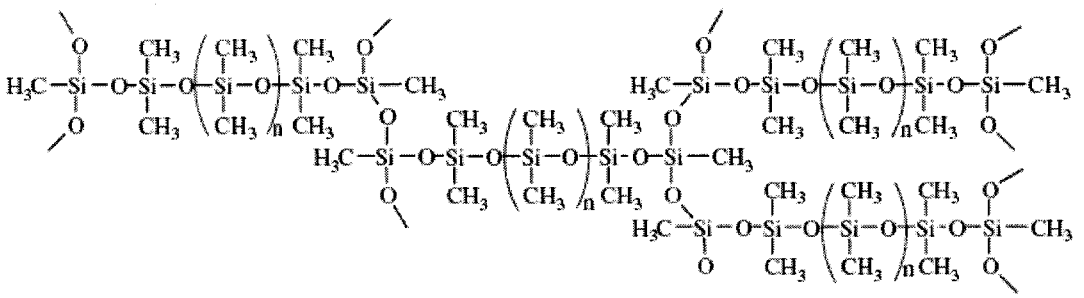
Under mild acid or base conditions, terminal silanol groups of polydimethylsiloxanes cause the polymer to be susceptible to condensation. A two stage condensation cure reaction is used to crosslink the polymer using moisture sensitive multi-functional silanes. The silanol is initially reacted with an excess of multi-functional silane resulting in the formation of a silicone with two end groups that are susceptible to hydrolysis. This process is shown in scheme 2-5 for an acetoxy system.

Scheme (2-5):



To complete the reaction, the end groups are exposed to moisture resulting in rapid crosslinking (scheme (2-6)).

Scheme (2-6) (from ref. 69):



2.5.2 PDMS Surface Modification

A lack of reactive functional groups on the PDMS surface has previously limited the options for PDMS surface modification to techniques that involve generation of a functional layer. Several strategies have been used to achieve this including the use of a mercury lamp to create radicals[136], oxidation by an O_2-based plasma to give alcohols and more highly oxidized species[137], and argon microwave plasma treatment[138], in order to generate surface functional groups for subsequent modification. However, these methods can be laborious, do not always produce consistent results, often result in incomplete surface coverage with the target functional molecule and are limited by the highly surface active nature of PDMS which tends to lead to reorientation of the polymer.

2.5.3 PDMS for Blood-Contacting Applications

Like many hydrophobic polymers, PDMS adsorbs significant quantities of proteins in the presence of blood, leading to coagulation. Therefore, the surface of silicone rubber polymers needs to be modified to improve their biocompatibility and performance for blood contacting applications. Efforts for modifying silicone polymers have included treating the surfaces by plasma polymerization of hydrophilic monomers[70] and UV-ozone[71]. However surfaces prepared in this fashion are often inconsistent and deteriorate rapidly over time. Protein coating is another common treatment for modifying the surface of silicone elastomers. Cell adhesive proteins, including fibronectin[72] and collagen[73] have been used with some success to promote cell adhesion on PDMS. However, the use of proteins for surface coatings is limited for long term applications due to proteolysis of the protein over time, loss of surface coating over time, and protein denaturation[74,75]. Other modification techniques include surface chemical treatments that permanently modify the surface properties of silicones and surface modification by covalent attachment of suitable macromolecules. Highly biocompatible PDMS surfaces may be produced by attaching biological molecules in order to render the material "bioactive". This may be done by means of direct attachment of the molecule to the surface, or by attachment via organic polymer chains. Therefore, covalent modification with amino acids, cell adhesion peptides, proteins, and glycosaminoglycans is possible[76].

2.6 Biomaterial Surface Modification with Polyethylene Oxide (PEO).

Polyethylene oxide is an uncharged, crystalline, thermoplastic polymer with the chemical formula, $(-CH_2CH_2O-)_n$. The polymer is characterized by its linearity, hydrophilicity, and high water solubility. PEO is completely miscible with water, though its solubility decreases with increasing temperature[77]. The presence of bulky side groups attached to a polymer backbone will introduce steric hindrances, reducing the probability of conformational transitions and reducing the mobility of polymer segments[78]. PEO is free of bulky side groups and rotation of the chain around the C-O bond of the ether is relatively facile even in comparison to an unsubstituted C-C bond. As a result, PEO is a polymer of very high chain mobility and flexibility.

It has been widely demonstrated that PEO modified surfaces have increased resistance to protein adsorption[8-11]. Several explanations have been proposed for PEO's protein repellant properties including its minimal interfacial free energy with water, high surface mobility, hydrophilicity, steric stabilization effects, and unique solution properties[8,79]. Jeon and Andrade[80] suggested that when diffusion promotes a collision of a protein with the PEO layer, van der Waals attractions between PEO and the protein, and between the protein and the substrate beneath the PEO layer occur resulting in compression of the PEO layer. The resulting steric repulsion caused by the compression of the PEO layer may therefore play a major role in resisting protein adsorption to the substrate. The level of resistance is dependent on the density and thickness of the PEO layer. It was also shown that the refractive index of PEO is low, resulting in low van der Waals attraction with the protein when compared to other water-soluble synthetic

polymers[81]. Lee et al.[82] suggested that hydrated PEO chains have a large excluded volume and are highly mobile in water thereby repelling protein molecules that approach the surface (Figure 2.7). PEO's highly miscible nature in water compared to other polyethers is believed to be related to hydration of the ether oxygens in a manner which is unique to the PEO structure[83,84]. Kjellander and Florin[85] have suggested that the water solubility of PEO results from a good structural fit between water and the polymer. In contrast to the other polyethers, PEO can be fitted into the tetrahedral water lattice so that all the lattice points are occupied either by a water or an ether oxygen. The ethylene segments may then fill out the voids in the water structure thereby causing minimal structural perturbation. Rapidly moving hydrated PEO chains on a surface effectively prevent adsorption of proteins on the surface by means of the formation of a hydration layer and a large excluded volume. Several studies have concluded that the conformation and resulting hydration state of surface grafted PEO, as determined by chain density and molecular weight, is largely responsible for inhibiting protein adsorption to these surfaces[86]. Optimal protein repulsion has been noted at intermediate surface grafted PEO densities[11]. Grafting density itself may be limited by PEO molecular weight due to steric restrictions encountered during the addition of chains to partially covered surfaces[11].

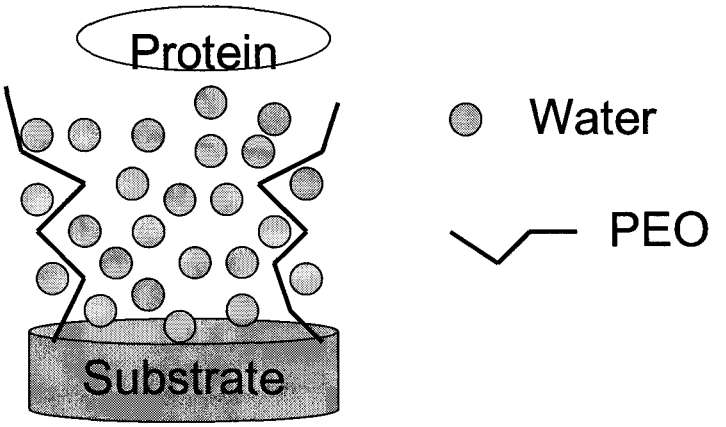


Figure 2.7. Schematic representation of surface-bound hydration layer. (from ref. 86).

Several approaches have been explored to immobilize PEO to surfaces including covalent attachment[87], chemisorption[87], and crosslinked network formation[88]. PEO has also been covalently grafted to surfaces in various conformations including comb[89,90], star,[91] and linear conformations[92]. Merrill[93] achieved increased PEO densities using a divinyl benzene core from which 10 to 50 PEO chains extend. Du and Brash[87] showed that HSA adsorption was significantly decreased on star PEO modified surfaces and that protein adsorption decreased as the number of branches increased. Therefore, several parameters affect the protein repellant properties of surface grafted PEO including surface chain density, conformation, and molecular weight.

2.7 Cell-Seeded Vascular Grafts

Synthesis of polymeric graft materials has focused on two fundamental properties that are critical to the success of vascular graft prosthetics: thrombogenicity and mechanics. Ideally, development of a fully biological arterial graft would satisfy both

criteria by possessing an anticoagulant endothelial cell layer and visco-elastic properties matching those of native vessels. Weinberg and Bell[94] demonstrated the possibility of creating a functional blood vessel substitute using a three layer technique comprised of natural materials. Fibroblasts and collagen were used to create an adventitia-like layer, SMC's and collagen formed a media-like layer, and ECs were used to create an intima-like monolayer. Finally, the entire construct was given a tubular configuration. L'Heureux and Auger et al.[95] succeeded in increasing the mechanical strength of such constructs by culturing them over a central mandrel. Recently, Seliktar et al.[96] have reported significant improvement in vessel strength as a result of dynamic mechanical stimulation which leads to collagen restructuring. However, the final product remained incapable of withstanding arterial pressures. Though it may ultimately be favorable to develop a fully biological blood vessel substitute, this technique remains limited by poor mechanical properties, insufficient three dimensional culture capabilities, immunological incompatibility, and lack of off-the-shelf availability[97].

One promising technique for circumventing undesirable blood-biomaterial interactions while maintaining a graft's mechanical integrity entails seeding ECs onto the inner surface of a synthetic graft. This approach exploits both the physiological anticoagulant characteristics of the endothelium and the desirable physical properties of synthetic materials. There are two types of seeding procedures. The first, known as single-stage seeding, involves the harvesting of ECs for immediate seeding onto the graft during the surgical procedure. The second procedure, known as two-stage seeding, involves harvesting of ECs followed by prolonged cell culture to increase the number of

available cells. Once sufficient cells have been cultured they are seeded onto the luminal surface of the polymeric graft for subsequent implantation. Two-stage seeding is preferable as it allows a large number of ECs to be seeded while single stage seeding generally results in low density luminal coverage resulting in limited graft patency in clinical trials[98] (Figure 2.8).

Authors	Type of seeding	Clinical use	Source of EC	Number of seeded grafts	Patency/years
Meinhart et al. [187]	Two	PVBG	Vein	153	84% at 4 years
Laube et al. [50]	Two	CABG	Vein	14	91% at 2.5 years
Deutsch et al. [70]	Two	PVBG	Vein	113	65% at 9 years
Williams [188]	Single	PVBG	Fat	11	60% at 4 years
Lesoche et al. [189]	Two	PVBG	Vein	21	67% at 6.3 years
Herring et al. [62]	Single	PVBG	Vein	66	38% at 2.5 years
Moorbaum et al. [190]	Single	PVBG	Fat	34	42% at 2.5 years

Figure 2.8. Major clinical trials of single and two-stage seeded vascular grafts, (from ref. 34).

At present, attempts to seed ECs on biomaterials have proven to be difficult due in large part to the low number of ECs that remain on the surface upon exposure to fluid shear stress similar to that found in arterial circulation[99]. Rosenman et al.[100] showed that within the first 30–45 min of blood flow, up to 70% of the initially seeded cells are lost from ePTFE grafts. In a study by Salacinski et al.[98] some ePTFE grafts have shown EC attachment of only 10±7% of applied cells, with only 4±3% of the ECs retained under flow. As a result, long term patency of EC seeded grafts has been limited. Therefore, much effort has been spent on improving EC adherence to biomaterial scaffolds.

2.8 RGD Peptides for enhanced cell adhesion

One approach for improving cell retention is to design a biomaterial that can directly exploit the properties of extracellular matrix (ECM) macromolecules in order to induce and direct desirable cellular processes. Therefore, in order to improve cell adhesion to a synthetic substrate, cell adhesion peptides containing the adhesive arginine-glycine-aspartic acid (Arg-Gly-Asp or RGD) sequence (Figure 2.9) derived from ECM proteins such as fibronectin and vitronectin have been grafted to material surfaces[17]. Walluscheck et al.[101] found a significant increase in cell adhesion and retention on ePTFE grafts coated with RGD peptide when compared to grafts coated with fibronectin and uncoated grafts under flow conditions.

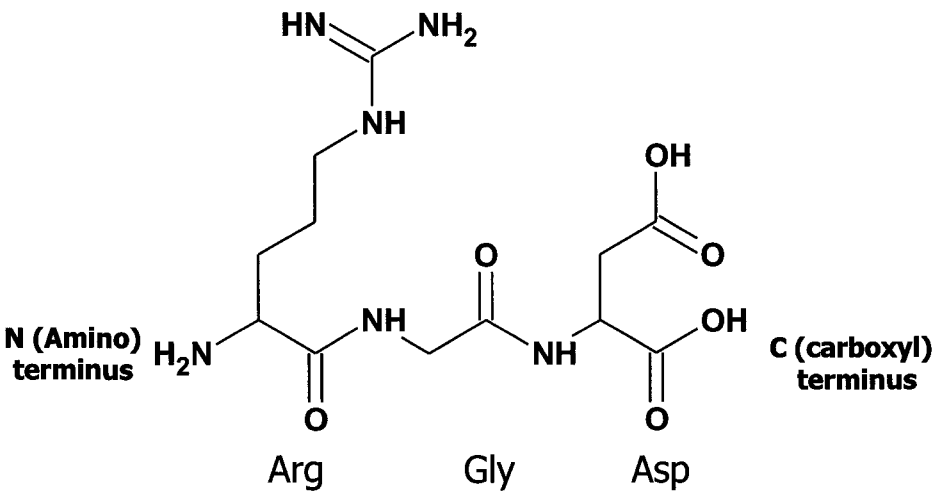


Figure 2.9. Chemical structure of Arg-Gly-Asp (RGD) cell adhesion peptide.

Other cell adhesive sequences and RGD variants have also been used successfully for increasing cell attachment to surfaces[102]. Delforge et al. found further enhancement of cell attachment by employing RGD cyclopeptides[103]. Xiao and Truskey reported that the use of immobilized cyclic RGD peptides leads to higher shear stress cell detachment resistance compared to linear peptides[104].

Manipulation of cell–integrin interactions with bioactive ECM ligands covalently grafted on synthetic biomaterials has shown great promise for improving cell adhesion and spreading on biomaterial scaffolds. It has also been found that such interactions are mediated by cell surface integrin clustering leading to maximum cell signal transduction and response[105,106]. Furthermore, there is growing evidence that bioactive ligand mobility is an important factor for effective interaction with such cell integrin clusters[29,107,140]. Thus, rather than being immobilized directly to a synthetic surface, ligands may be attached to a substrate via flexible organic polymer tethers such as polyethylene oxide in an attempt to improve ligand-receptor interactions between the polymer surface and cells. Griffith et al.[31] showed that the degree of cell adhesion on a PEO crosslinked hydrogel was strongly influenced by the degree of cell adhesion peptide mobility as determined by PEO tether length. The extent of hepatocyte cell spreading was found to be dependent not only on the surface concentration of galactose, for which hepatocytes express a surface binding receptor, but also on its ability to cluster into microdomains. It has also been shown that cell motility on a biomaterial surface can be regulated by modifying the magnitude of cell-substrate adhesion[108,109]. Mann and West reported that migration and proliferation of smooth muscle cells were lower at elevated surface concentrations of

adhesion peptides[110]. Cell morphology[111], attachment and extra-cellular matrix production[113] have been shown to vary with surface peptide concentration and spacing for a number of different cell types. Various polymeric structures have also been employed for attachment of cell adhesion peptides to biomaterial surfaces in order to control the distribution and density of surface bound peptides including comb[114] and star [115] conformations. Varying the structural presentation of surface grafted peptides has led to the belief that both peptide clustering and spatial orientation may also be important control mechanisms for cell adhesion and motility.

2.9 Dendrimer Polymers

Dendrimers are highly ordered oligomeric and polymeric compounds formed by reiteration reaction sequences starting from smaller molecules or "initiator cores"[119]. These highly branched molecules are constructed using identical monomeric building blocks to create branching sites located in a spherical manner around a core (Figure 2.10). The addition of consecutive monomer shells is referred to as generations, with the number of terminal functional groups doubling (or tripling) for each generation. Dendrimer synthesis is highly controllable and results in macromolecules of good structural precision. As a result, dendrimers play an important role in the rapidly growing field of supramolecular chemistry. Dendrimers possess unique three dimensional structural properties unlike those of linear polymers which tend to possess random-coil configurations. At higher generations, dendrimers may act as "unimolecular" micelles capable of incorporating molecules within their interior spaces referred to as "dendritic

voids". Dendrimers combine characteristics that are typical of small organic molecules, such as monodispersity and highly defined structures, with attributes that are typical of traditional high molecular weight synthetic polymers. Unique surface properties of dendrimers may incorporate variations in shape, reactivity, and kinetic features and can allow for surface-specific recognition capabilities by attachment of targeting groups[120]. The unique properties of dendrimers have resulted in their use in various applications including molecular encapsulation, light-harvesting systems, catalysis, and biomedical applications[121].

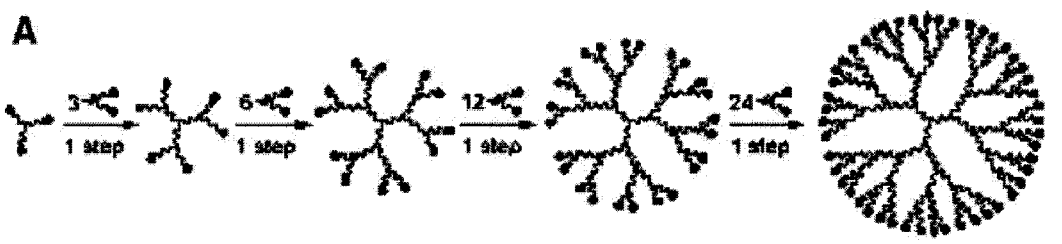


Figure 2.10. Generic structure of a dendrimer generated by divergent synthesis, generations 1 to 4. From [122]

2.9.1 Dendrimers in Biomedical Applications

The formation of molecular systems with well defined structures and sizes is of great interest for use in various biomedical applications such as drug delivery, gene transfection, and imaging. The high level of control possible over dendrimer structure, size, shape, branching length and density, and surface functionality, makes dendrimers

ideal for these applications. For example, bioactive agents may be encapsulated into the interior of dendrimers or chemically attached or adsorbed onto the dendrimer surface[123]. Also, the high density of surface functional groups provides the opportunity to tailor the properties of the dendrimer carrier to the specific needs of various therapeutic applications[124]. For example, the attachment of targeting groups or specific functionality to the dendrimer surface may allow for control over its solution behavior, toxicity, and cellular interactions[120].

Dendrimers have been intensely investigated a delivery vehicles for DNA and small organic molecule drugs in applications such as cancer therapy. Recently, amino-terminated PAMAM dendrimers have been used as non-viral gene transfer agents by enhancing the transfection of DNA by endocytosis and migration into the cell nucleus[125]. Ohsaki et al.[126] has developed dendritic poly(l-lysine)s at several generations in order to compare the gene transfection properties against linear and branched poly(lysine) architectures for peptide based molecules. They found that third generation and higher dendrimers could form a complex with a DNA plasmid and that the degree of DNA compaction increased with increasing dendrimer generation. Therefore they concluded that gene transfection efficiency could be determined as a function of dendrimer generation.

In addition to DNA, dendrimers have been utilized to carry a variety of small molecule pharmaceuticals. For example, encapsulation of the anticancer drug cisplatin within PAMAM dendrimers resulted in slow drug release, higher accumulation in solid tumors, and lower toxicity compared to free cisplatin[127]. Dendrimers can also be used as

building blocks for use in controlling the solubility of various pharmaceuticals. This has been accomplished by covalently connecting two polyester dendrons and using one of the dendrons to provide multiple functional groups for the attachment of drug molecules, and the other dendron to attach high solubility poly(ethylene oxide) chains. By varying the generations of dendrons and the mass of the PEO chains, drug loading capacity can be readily controlled[128,129]. Kojima et al.[130] created polyamidoamine dendrimers having poly(ethylene glycol) grafts as a novel drug carrier which possesses an interior for the encapsulation of drugs and a biocompatible surface. Their ability to encapsulate these drugs increased with increasing dendrimer generation and chain length of poly(ethylene glycol) grafts (Figure 2.11).

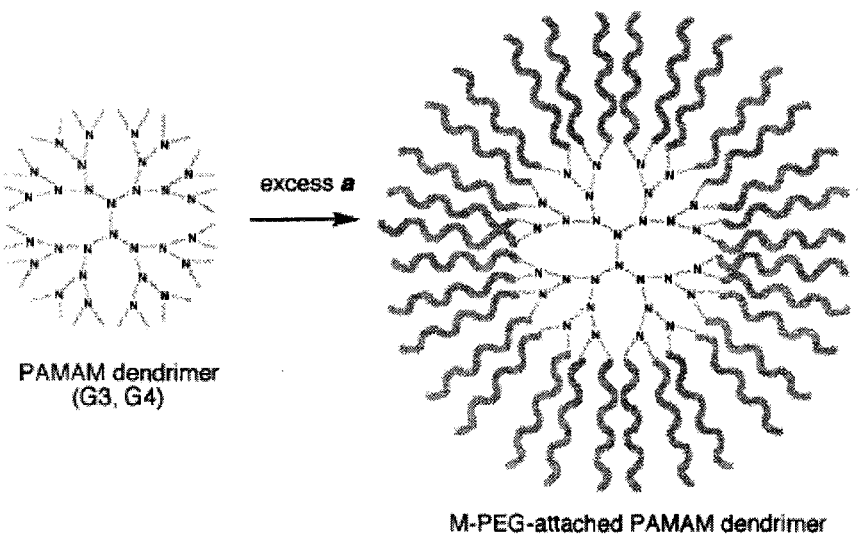


Figure 2.11. Synthesis of polyamidoamine dendrimers containing surface grafted poly(ethylene glycol) for increased biocompatibility for encapsulation of anticancer drugs, (from ref. 130).

Dendrimers have also received increasing attention for their use as nano-scaffolds. The surface of dendrimers provides an ideal means for the attachment of cell-specific ligands, solubility modifiers, and organic molecules capable of guiding the interaction with macromolecules present in the body. One example of cell-specific dendritic carriers is a dendrimer which incorporates surface bound folic acid for targeting a variety of cancer cells that over-express a membrane folate receptor. These folate-modified dendrimers were internalized into some cancer cells preferentially over normal cells via receptor-mediated endocytosis for delivery of cytotoxic substances[131]. Witvrouw et al.[132] found that PAMAM dendrimers containing surface grafted naphthyl sulfonate residues exhibited antiviral activity against HIV. The resulting dendrimer-based nano-drug inhibited early stage virus adsorption on cells and later stage viral replication. The mechanism of its action was determined to be via interference with reverse transcriptase and/or integrase enzyme activities.

Dendrimers have also been explored for use in biomedical imaging applications. Paramagnetic metal chelates and their derivatives increase the relaxation rate of nearby water protons and are used as contrast agents for magnetic resonance imaging (MRI). However, these low molecular weight contrast agents have short circulation times within the body and are often inefficient at discriminating between diseased and normal tissues[120]. Wiener et al. used dendrimer gadolinium poly-chelates as MRI contrast agents for increasing circulation times and achieving high proton relaxivities. These dendrimer-

metal chelate conjugates were found to enhance conventional MR images and 3D time of flight MR angiograms[133].

Due to the breadth of unique and customizable properties associated with dendrimers, these polymers may play a significant role in the development of a variety of new biomedical technologies.

3 Research Objectives

Biological responses at the blood-material interface, including non-specific protein adsorption, coagulation, and platelet adhesion and activation, significantly limit the use of current biomaterials in blood contacting applications. Several strategies have been used to modify the surface of biomaterials including passivating the material such that the tissue-material interface is resistant to non-specific protein adsorption, the initial adverse host response to the foreign implant. More recently, bioactive materials have been designed which induce desirable chemical and cellular responses *in vivo* by stimulating specific interactions with desired proteins and cells. The proposed research will therefore target the design, synthesis, and characterization of PDMS based material surfaces capable of increasing cell adhesion by surface grafting of cell adhesion peptides via PEO linker molecules.

Specific hypotheses include:

- PEO may be used as an effective tether molecule for attachment of cell adhesion peptides to PDMS. The resulting mobility of the tethered ligands will increase ligand clustering capabilities necessary for increased cell signal transduction.
- The use of PEO as a linker molecule for the attachment of cell adhesion peptides will reduce non-specific protein adsorption while increasing cell adhesion.
- Peptide grafting density on the biomaterial surface will be controlled based on the reaction solution concentration of the peptide.

- Cell adhesion will increase with increasing surface peptide density until an 'ideal' or 'critical' surface peptide density for cell adhesion is observed.
- Surface grafted dendrimers will increase biomaterial surface functionalization. As a result, higher grafting densities of cell adhesion peptides will be achieved.

Specific project objectives are as follows:

- Graft polyethylene oxide (PEO) to PDMS by means of a novel coupling chemistry. Characterize and optimize surface modification techniques.
- Covalently bind cell adhesion peptides to PDMS, via PEO, in order to promote vascular endothelial cell adhesion.
- Quantify cell adhesion on cell adhesion grafted PDMS surfaces.
- Exploit properties of dendrimer polymers to increase biomaterial surface ligand grafting potential.
- Compare cell adhesion on surfaces containing cell adhesion peptides bound by a PEO spacer to that on surfaces containing cell adhesion peptides bound by a dendrimer.

4 MATERIALS AND METHODS

4.1 Materials and Reagents

The materials and reagents used in the synthesis and characterization of PEO modified PDMS and their sources are summarized in Table 4.1.

Table 4.1. List of materials and reagents.

Material	**Source**
Poly(ethylene glycol) monoallylether, 550 Da.	Clariant Corp. (Markham ON, CA).
N,N'-disuccinimidyl carbonate	Sigma Aldrich®, Oakville, ON. CA
triethylamine (99%)	Sigma Aldrich®, Oakville, ON. CA
acetonitrile (99%, anhydrous),	Sigma Aldrich®, Oakville, ON. CA
Karstedt's Pt catalyst (2-3 wt% in xylene, platinum-divinyltetramethyldisiloxane, $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$)	Sigma Aldrich®, Oakville, ON. CA
Trifluoromethanesulfonic acid, (*triflic acid*), ≥99%	Sigma Aldrich®, Oakville, ON. CA
Diethylene glycol dimethyl ether, (*2-methoxyethyl ether*)	Sigma Aldrich®, Oakville, ON. CA
Silicone Elastomer Kit (Sylgard 184 curing agent and silicone elastomer base).	Dow Corning®, Midland, MI. U.S.A.
Poly(methylhydrogen siloxane), $(MeHSiO)_n$, (DC1107).	Dow Corning®, Midland, MI. U.S.A.
Gly-Tyr-Arg-Gly-Asp-Ser (GYRGDS) peptide, >99% purity.	American Peptide®, Sunnyvale, CA. U.S.A.
Arg-Gly-Asp-Ser (RGDS) peptide, >95% purity.	Sigma Aldrich®, Oakville, ON. CA
Arg-Asp-Gly-Ser (RDGS) peptide, >95% purity.	Sigma Aldrich®, Genosys, Oakville, ON. CA
Methanol (anhydrous)	Sigma Aldrich®, Oakville, ON. CA
Hexane	Sigma Aldrich®, Oakville, ON. CA
Trifluoroacetic acid	Sigma Aldrich®, Oakville, ON. CA
Heparin	Sigma Aldrich®, Oakville, ON. CA
Endothelial cell growth supplement	Sigma Aldrich®, Oakville, ON. CA
Trypsin-EDTA	Invitrogen®, Burlington, ON, CA
Fetal bovine serum	Invitrogen®, Burlington, ON, CA
Modified F-12K Culture Medium	American Type Culture Collection (ATCC), Manassas, VA.
Iodogen ®	Pierce, Rockford, IL, U.S.A
Radioactive sodium iodide $Na^{125}I$	ICN Pharmaceuticals, Irvine, CA, U.S.A
Fibrinogen	Calbiochem, La Jolla, CA, U.S.A

4.2 Preparation of PDMS Disks

Sylgard 184 elastomer base was mixed thoroughly with its curing agent in a 10:1 (w/w) ratio and poured into a polystyrene dish. The polymer film was degassed under vacuum for 2 hours and left to cure for a minimum of 2 days at room temperature. After curing, the PDMS film was punched into disks 8mm in diameter and approximately 0.5mm thick.

4.3 Surface Functionalization of PDMS Disks

Si-H functional groups were created on the PDMS surfaces in order to allow for subsequent PEO grafting (Figure 4.1).

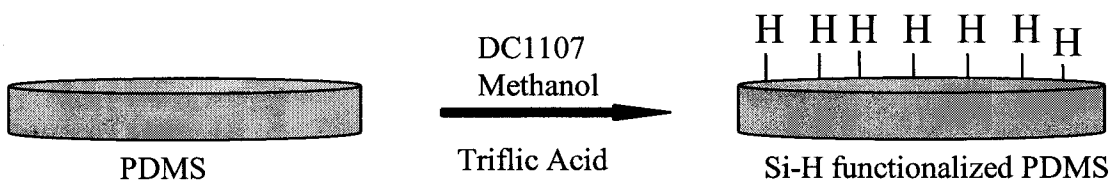


Figure 4.1. Generation of surface Si-H functional groups on PDMS disks

Thirty PDMS disks were placed in a solution of $(MeHSiO)_n$ (DC1107) (Figure 4.2) and methanol (DC1107:methanol, 3:5 (v/v)) containing triflic acid as catalyst (2% (v/v) triflic acid in methanol). The solution containing the disks was shaken for 30 minutes on a rotating shaker at ~250 RPM. The PDMS disks were then removed and washed thoroughly with anhydrous methanol (3x5mL), hexane (1x10mL), and again with

anhydrous methanol (1x5mL). The disks were dried by N_2 and placed under vacuum for 8 h at room temperature.

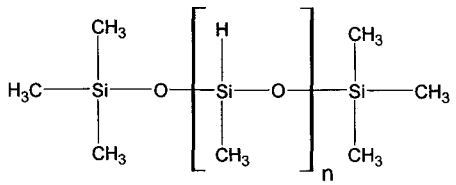


Figure 4.2. Chemical structure of polymethylhydrogen siloxane, $(MeHSiO)_n$, (DC1107).

4.4 Preparation of PEO-grafted PDMS Disks

4.4.1 Method 1 - Direct Grafting of allyl-PEO-NSC to PDMS

4.4.1.1 Synthesis of α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol) , (allyl-PEO-NSC)

N,N'-disuccinimidyl carbonate (4.1g, 16 mmol) was added to a solution of poly(ethylene glycol) monoallylether MW 550 (2.0g, 4 mmol), triethylamine (1.62g, 16 mmol) and anhydrous acetonitrile (Figure 4.3).

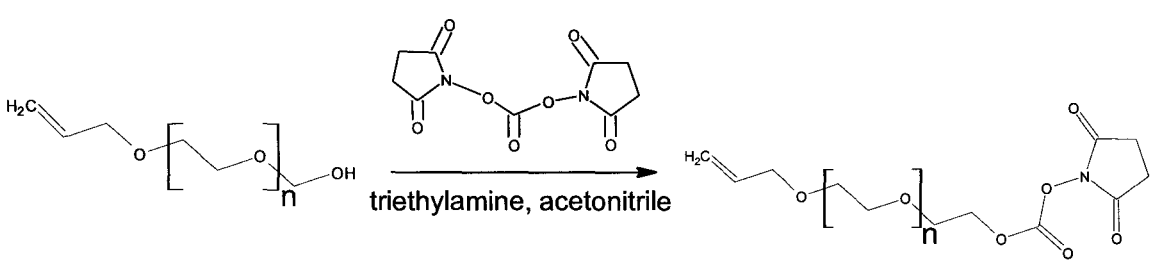


Figure 4.3 Synthesis of α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol), (allyl-PEO-NSC).

The mixture was stirred at room temperature for 10 h under N_2. After removal of the solvent in vacuo, the residue was dissolved in dry toluene (25mL) and the solution was

cooled to below 0°C in the freezer for 12 hrs. The toluene solution was decanted into a clean flask leaving behind a pale brown precipitate which was discarded. The solution was then removed in vacuo leaving a viscous yellow liquid. Toluene was added and the solution cooled and evaporated 6 times or until a purity of at least 8:1 allyl-PEO-NSC (Figure 4.4) : NHS (Figure 4.5) was achieved by ^{1}H-NMR spectroscopy. The resulting product was a viscous yellow oil (~60% yield).

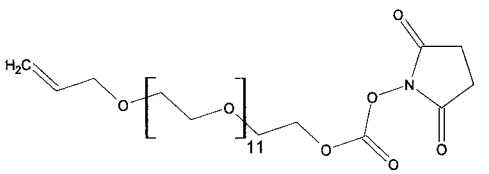


Figure 4.4. α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol), (allyl-PEO-NSC).

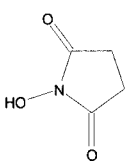


Figure 4.5. *N*-hydroxysuccinimide (NHS).

4.4.1.2 Attachment of allyl-PEO-NSC by Hydrosilylation

Twenty Si-H modified PDMS disks were placed in a solution (3mL) containing 0.6 g allyl-PEO-NSC in a 20 mL glass scintillation vial (Figure 4.6). Platinum catalyst (15 μL) was added and the mixture stirred for 20 h at 50°C. The PEO-NSC modified PDMS disks were then removed and washed thoroughly with acetone (3x5mL), dried by N_2 and placed under vacuum for 12 h.

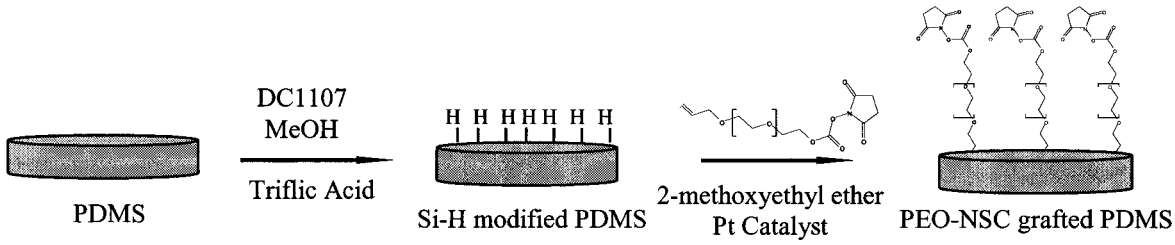


Figure 4.6. Surface grafting of allyl-PEO-NSC to PDMS via a hydrosilylation reaction.

4.4.2 Method 2 - Grafting of allyl-PEO-OH to PDMS Surface by Hydrosilylation and Subsequent Functionalization with NHS Ester

4.4.2.1 Grafting of allyl-PEO-OH to PDMS

Ten Si-H modified PDMS disks were placed in a solution of 2-methoxyethyl ether (3mL) containing 0.3mL poly(ethylene glycol) monoallylether, 500 Da, (allyl-PEO-OH) in a 20 mL glass scintillation vial (Figure 4.7). To this 15 μL of Pt-catalyst was added and the mixture was shaken on a rotating shaker (~150 rpm) for 2 h at room temperature. The PEO-OH modified PDMS disks were then removed and washed thoroughly with acetone (3x5mL), dried by N_2, and placed under vacuum for 12 h. The reaction scheme is summarized in Figure 4.8.

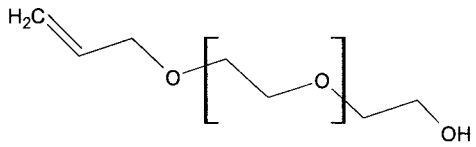


Figure 4.7. Chemical structure of poly(ethylene glycol) monoallylether (allyl-PEO-OH).

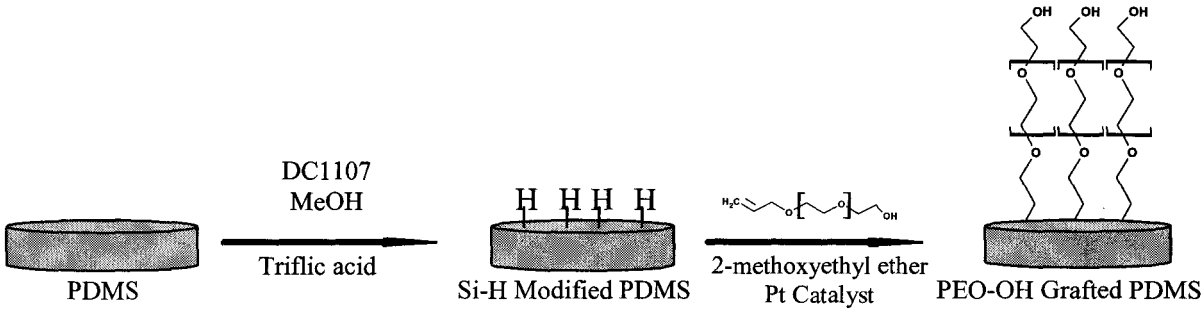


Figure 4.8. Surface grafting of allyl-PEO-OH to PDMS via a hydrosilylation reaction.

4.4.2.2 Functionalization of Surface Grafted PEO-OH with NHS Ester.

Ten PEO-OH modified surfaces were placed in a solution of acetonitrile (1mL), triethylamine (0.1mL), and *N,N'*-disuccinimidyl carbonate (0.2g) in a 20 mL glass scintillation vial (Figure 4.9). The reaction vial was purged with nitrogen and shaken for 6 h on a rotating shaker at 150RPM. The disks were then washed with acetonitrile (2x5mL), 2-methoxy ethyl ether (1x5mL), again with acetonitrile (1x5mL) and subsequently dried using nitrogen and placed under vacuum for 12 hours.

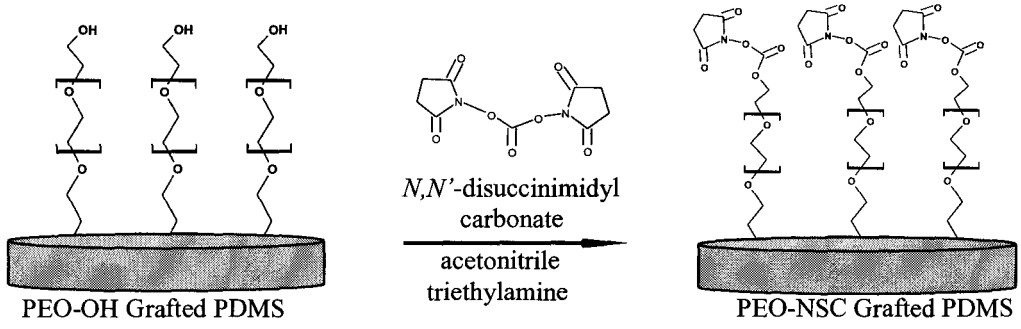


Figure 4.9. Generation of NHS ester functional group at terminus of surface grafted PEO.

4.5 Grafting of diaminobutane (DAB) Dendrimer to PDMS

Eight PDMS disks modified with surface grafted PEO-NSC by method 2 were placed in a solution containing diaminobutane dendrimer G3 (0.03g, 17.8μmol) in dichloromethane (CH_2Cl_2) (2mL) and the 20 mL glass scintillation reaction vial was shaken using a rotating shaker for 6 h at 150RPM (Figure 4.10). The disks were then washed in CH_2Cl_2 (3x1mL), dried using nitrogen and placed under vacuum for 12 h. The amount of dendrimer in the reaction solution was 16 fold molar excess relative to available terminal NSC ester groups per disk. Using the maximum surface peptide density achieved by the PEO-NSC linker system, as determined by radiolabeling with ^{125}I, the amount of surface NSC groups was estimated by assuming a 1:1 (PEO-NSC:peptide) reaction resulting in the consumption of all available NSC groups. Dendrimer was added in excess such that the theoretical number of PEO chains that may have attached to any single dendrimer molecule was minimized in order to achieve maximum availability of terminal amine groups for subsequent peptide grafting.

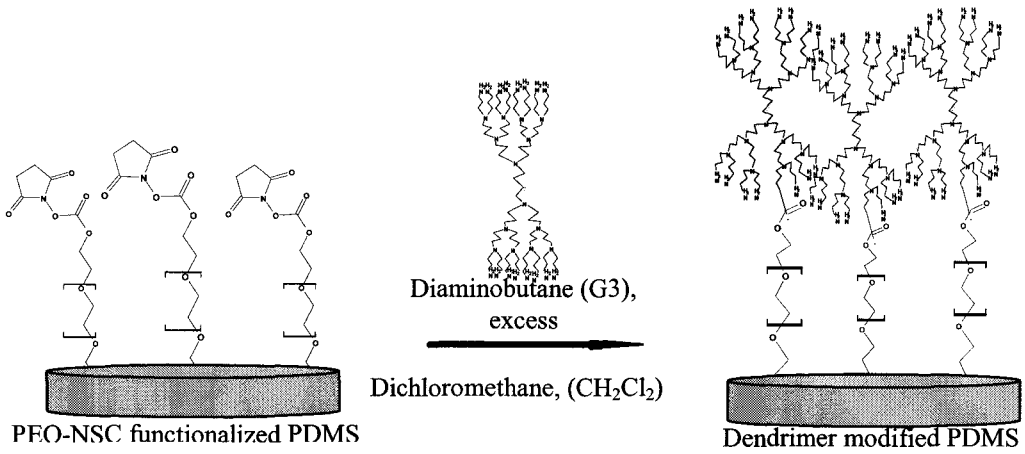
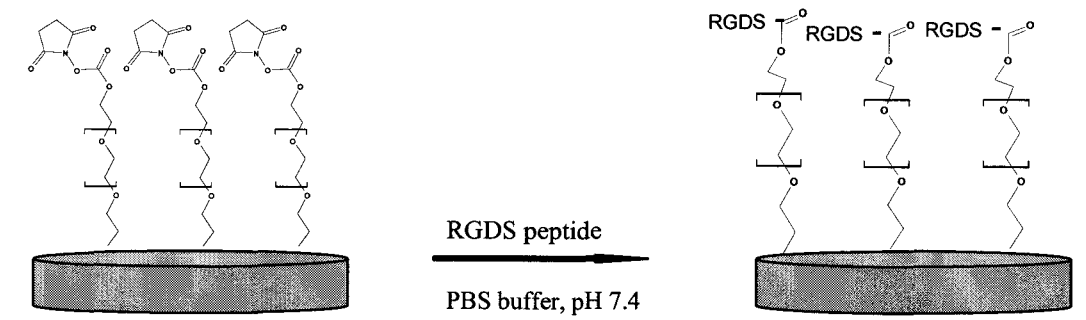


Figure 4.10. Grafting of diaminobutane dendrimer (G3) to PDMS surface.

4.6 Attachment of Cell Adhesion Peptides to PEO-NSC Modified PDMS

Cell adhesion peptide RGDS or GYRGDS was dissolved in PBS buffer (pH 7.4) and placed in the wells of a 48 tissue culture well-plate at desired concentrations. PDMS disks modified with PEO-NSC by method 1 or 2 were placed in the wells containing the peptide solution and allowed to soak for 2 hours (Figure 4.11). The disks were then rinsed in wells containing PBS buffer 3 times for 10 minutes. Attachment of the peptide to the PEO-NSC modified disks occurs via aminolysis by the amine terminus of the peptide of the NHS ester located at the terminus of the surface grafted PEO chain. This reaction produces an amide linkage between the peptide and the surface bound PEO.



PEO- NSC functionalized PDMS PEO-RGD functionalized PDMS

Figure 4.11. Covalent attachment of RGDS cell adhesion peptide to PDMS via PEO linker.

4.7 Attachment of Cell Adhesion Peptides to Dendrimer Modified PDMS

Attachment of GYRGDS or RGDS peptides to dendrimer modified disks was achieved by first converting the carboxylic acid groups of the peptide to reactive NHS esters using 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and NHS. A molar ratio of 5:5:1 (EDC:NHS:COOH of peptide) was used for reaction of EDC and NHS with the peptide in PBS buffer (pH of 5.5) for 24 hours. Subsequent dilutions were conducted in order to achieve desired peptide solution concentrations. The peptide was immobilized to the dendrimer via a reaction of the peptide NHS-ester with primary amino groups on the surface grafted dendrimer.

Molecules containing a carboxylic acid group react with EDC to form an amine-reactive intermediate. This intermediate may react with molecules containing an amine, yielding a stable amide linkage. However, the intermediate is also susceptible to hydrolysis, making it unstable and short-lived in aqueous solution. The efficiency of the EDC coupling reaction may be increased by the addition of NHS, which stabilizes the amine-reactive intermediate by converting it to an amine-reactive NHS ester. A general reaction scheme showing the use of EDC-NHS as a zero-length crosslinking agent for coupling of carboxylic acid groups to primary amines is shown in Figure 4.13.

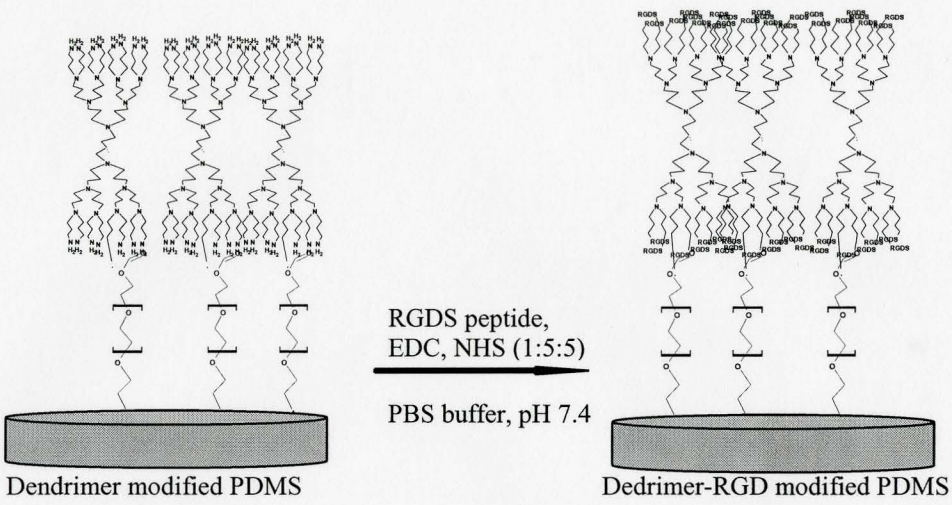


Figure 4.12. Covalent attachment of RGDS cell adhesion peptide to PDMS via PEO-dendrimer linker.

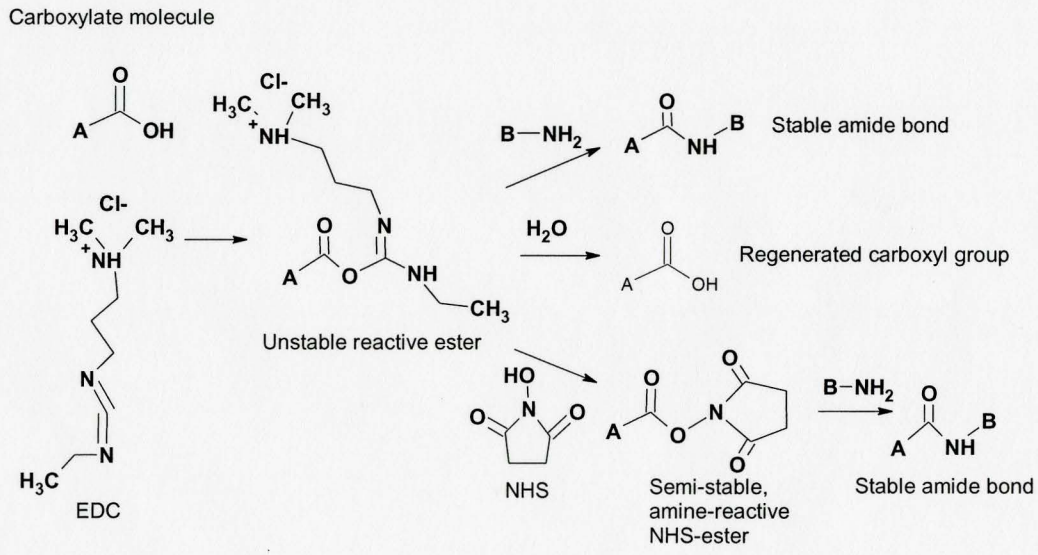


Figure 4.13. Coupling of carboxylic acid groups to primary amines via EDC and NHS, (from ref. 112).

4.8 Surface Characterization

4.8.1 Sessile Drop Contact Angles

In order to determine the relative surface hydrophilicity/hydrophobicity of the material surfaces, sessile drop contact angles were used for rapid and quantitative assessment. Samples were placed on glass slides. All materials were analyzed by placing a 10 μL drop of Milli-Q water on the surface and measuring the advancing and receding contact angles using a Rame-Hart NRL 100-00 goniometer (Mountain Lakes, NJ).

4.8.2 Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR)

Attenuated total reflection Fourier transform infrared spectroscopy allows for analysis of surface chemical composition by revealing the presence of chemical bonds at a depth of between 1 and 5 μm. Different functional groups result in different vibration and/or stretching frequencies when exposed to infrared (IR) radiation. Analysis of the absorbance spectra reveals information about the functional groups present. In this work, a Bruker TENSOR (Bruker Instruments, Billerica, MA), Fourier transform infrared spectrometer was used for analysis of all surface modifications.

4.8.3 X-ray photoelectron spectroscopy (XPS)

XPS was used to determine the quantitative elemental composition of the material surfaces. Analysis was performed at Surface Interface Ontario at the University of Toronto using a Leybold Max200 X-ray photoelectron spectrometer with a MgK non-

monochromatic X-ray source. Low and high resolution XPS measurements were made at takeoff angles of 90°, 30° and 20° in order to obtain compositional data at different depths. The spot size used in all cases was 2×4 mm. Data analysis was performed using Specslab software (specs Gmbh, Berlin).

4.8.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an analytical technique used for determining the molecular structure of a chemical sample. In this work, ^{1}HNMR was used to verify the synthesis and purification of α-allyl-ω-*N*-succinimidyl carbonate-poly(ethylene glycol). A Bruker AV200 pulsed NMR spectrometer operating at room temperature, 75.47 Hz, and a 10,000 Hz rotation rate was used for all measurements.

4.9 Radiolabeling Using ^{125}I

4.9.1 Labeling of Peptides

Radioiodination of proteins is an established and sensitive technique for the quantification of small amounts of protein or peptides. For successful ^{125}I radiolabelling, the protein to be labeled must contain amino acids possessing a sulfur atom or an aromatic structure (e.g. cysteine, histidine, methioninephenylalanine, tryptophan, and tyrosine). For quantification of peptide binding, the cell adhesion peptide with amino acid sequence Gly-Tyr-Arg-Gly-Asp-Ser (GYRGDS) was labeled using Na^{125}I using the IODO-GEN® (Pierce, Rockford, IL) method. The peptide was first dissolved in tris buffered saline (TBS) (pH 7.4) at a concentration of 1mg/mL. 10 μL of Na^{125}I was added

to a solution containing 250 μg of GYRGDS peptide in buffer. The solution was placed in an IODO-GEN® coated vial and stirred for 15 minutes. Separation of the peptide and unbound ^{125}I after iodination was accomplished using a reverse phase Sep-Pak C_{18} column (Waters, Mississauga, ON) containing a silica-based bonded phase of strong hydrophobicity. The column was pre-equilibrated with 6-10mL of methanol followed by 6-10mL of 0.1% trifluoroacetic acid (TFA) in TBS buffer at pH 7.4. The reaction solution was poured into the column and rinsed with approximately 15mL of 0.1% TFA in buffer. Solution fractions were collected in 1 mL aliquots and measured for radioactivity using a handheld gamma counter (Surveyor M, BICRON, Solon, OH). Following elution of the unbound ^{125}I, the bound and labeled GYRGDS peptide was eluted using a solution of acetonitrile and 0.1% TFA in TBS buffer (acetonitrile:0.1% TFA in TBS, 20:80 (v:v)). Solution fractions were collected at the bottom of the column in 0.5 mL aliquots and were measured for radioactivity. The peptide concentration of the most radioactive sample fractions was determined by measuring the solution absorbance at 280 nm using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). Sample radioactivity was measured using a gamma-counter (Wallac 1480 Wizard 3" Automatic, Perkin-Elmer Life Sciences, Turku, Finland).

4.9.2 Labeling of Fibrinogen

Fibrinogen was first dissolved in phosphate buffered saline (PBS) at a concentration of 10mg/mL. Fibrinogen was then labeled with ^{125}I using the ICl method in which the Fg was reacted in a 1:4 molar ratio with ICl reagent and 5 μL of $Na^{125}I$

(Appendix A). Free iodide was removed by passing the labeled protein solution through an AG 1-X4 column (Bio-Rad Laboratories, Hercules, CA, USA). Residual free iodide in the protein solution recovered from the column was determined by precipitation of the protein using trichloroacetic acid (Appendix B). Protein solutions containing less than 3% unbound ^{125}I were used for subsequent protein adsorption experiments to sample surfaces.

4.10 Fibrinogen Adsorption to Modified PDMS Surfaces

To assess protein adsorption characteristics of the modified surfaces, fibrinogen adsorption experiments were performed. ^{125}I labeled fibrinogen was mixed with unlabeled fibrinogen (1:20, labeled:unlabeled) at a total concentration of 1 mg/mL and serially diluted with PBS buffer (pH 7.4). Modified PDMS surfaces were incubated with fibrinogen in buffer for 3 hrs at room temperature (~22°C) in the wells of 96 well flat-bottom multi-well plates (Beckton Dickson Labware, Franklin Lakes, NJ). The surfaces were then rinsed three times for 10 min each in PBS, wicked onto filter paper, and transferred to vials for determination of radioactivity using a gamma counter. Radioactivity was converted to amounts of adsorbed protein by comparison to radioactive fibrinogen solutions of known concentration as determined using a UV spectrophotometer.

4.11 Culture of Human Umbilical Vein Endothelial Cells (HUVECS)

Human Umbilical Vein Endothelial Cells were purchased from The American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured in F-12K cell culture medium modified by ATCC to contain 2mM L-glutamine and 1.5 g/L sodium bicarbonate in 75 cm^2 vented flasks. To this medium was added 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement (ECGS), and 10% fetal bovine serum. Cells were incubated at 37°C in a 5% carbon dioxide gas phase. Medium was replaced every two days and the cells were subcultured at near confluence approximately once every 10 days. For long term storage, cells were placed in medium containing 5% (v:v) DMSO, placed in a freezer at -80°C for 24 hours, and stored in liquid nitrogen.

4.12 Cell Adhesion

Cells were removed from the culture flask using trypsin-EDTA and resuspended in culture medium. The cells were then seeded onto sample surfaces in cell culture wells at a desired cell density, as determined using a hemocytometer, in 40 μL droplets. The seeded surfaces were incubated for two hours to allow for cell adhesion at which time 250 μL of culture medium was added to each well. After 6 hours, the culture medium was carefully aspirated and the seeded surfaces were rinsed gently by adding sterile PBS buffer (3x250 μL). Cell number was determined using the CyQUANT ® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) and a fluorimeter (Fluoroskan Ascent FL, Thermo Electron Corp.).

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5 CONTRIBUTIONS TO ARTICLES

The following describes my contribution to the articles constituting Chapters 6-8. I was responsible for the refinement of the PEO surface modification, initially designed in Dr. Michael Brook's lab, and the conduct of all subsequent phases of the project, from the literature research and the planning of experiments through the data analysis. I performed all experiments independently, excluding XPS sample analysis for which I shadowed Dr. Rana Sodhi at the University of Toronto. I generated the first drafts of the papers and worked with my supervisors to perform subsequent revisions.

6 PAPER ONE: CELL ADHESION PEPTIDE MODIFIED PDMS VIA ROBUST GENERIC POLYETHYLENE OXIDE LINKER CHEMISTRY REDUCES PROTEIN ADSORPTION WHILE SUPPORTING CELL GROWTH

Authors: A.S. Mikhail, J.J. Ranger, L. Liu, K.S. Jones, H. Sheardown, M.A. Brook

Working Hypothesis:

A surface grafted heterobifunctional allyl-,OH-terminated PEO linker molecule can be used as a generic linker molecule for attachment of biomolecules while reducing protein adsorption to the silicone elastomer substrate.

Main Scientific Contributions:

1. Introduced a new and more refined method for attachment of amine-containing biomolecules via a generic PEO linker molecule.

2. Protein adsorption results demonstrated significant reductions in protein adsorption on PEO and PEO-peptide modified surfaces.

3. Cell adhesion peptides grafted to PDMS via the PEO linker were bioactive and resulted in increased support of endothelial cell growth compared to controls.

Cell Adhesion Peptide Modified PDMS via Robust Generic Polyethylene Oxide Linker Chemistry Reduces Protein Adsorption While Supporting Cell Growth

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Abstract

A robust surface modification procedure was developed for functionalization of poly(dimethylsiloxane) materials with biologically active groups. Oligopeptide RGDS was surface grafted to poly dimethylsiloxane elastomers via a heterobifunctional allyl-, OH- terminated PEO linker molecule in order to improve endothelial cell adhesion. Attachment of the linker molecule was achieved by means of surface functionalization using hydrosilanes, followed by hydrosilylation using the allyl-terminated PEO linker. Replacement of the terminal –OH group with the amine reactive NSC functional group permitted covalent attachment of amine containing peptides. ATR-FTIR, X-ray photoelectron spectroscopy (XPS), and water contact angle results confirmed the successful attachment of the polymer linkers while XPS also confirmed the presence of surface grafted peptides. Bioactivity of the grafted peptides was demonstrated by *in vitro* culture of endothelial cells on the modified substrates. Adsorption of ^{125}I radiolabeled fibrinogen to PEO and PEO-peptide modified surfaces demonstrated a significant reduction in protein adsorption compared to controls.

Keywords: PDMS, PEO, generic modification, cell adhesion peptide

Introduction

Poly(dimethylsiloxane) (PDMS) has many desirable properties that make it an attractive material for various biomedical applications [1]. However, its use is limited by the non-specific adsorption of proteins upon exposure to protein-containing solutions and the subsequent deleterious effects [2]. Non-specific adsorption of blood proteins has, for example, been widely demonstrated to mediate subsequent biological reactions including coagulation, and platelet adhesion and activation [3]. Adsorption of proteins from tears to the surface of contact lenses is associated with discomfort, lens spoliation and other adverse effects. Therefore it is desirable to modify the surface of PDMS with biologically relevant molecules in order to mitigate these adverse reactions.

A lack of reactive functional groups on the PDMS surface has previously limited the options for PDMS surface modification to techniques that involve generation of a functional layer prior to surface grafting [4-6]. However, these methods can be laborious, are not highly reproducible, often result in incomplete surface coverage with the target functional molecule and are limited by the highly surface active nature of PDMS which tends to lead to reorientation of the surface and exposure of the PDMS layer.

PEO, a water-soluble, nontoxic, and non-immunogenic polymer that is highly mobile in aqueous solution, has been widely shown, when present on a biomaterial surface, to lead to a reduction in nonspecific protein adsorption [7-11]. In order to reduce protein

adsorption on PDMS surfaces, we have previously incorporated monofunctional and homobifunctional PEO chains into the polymer structure during the vulcanization process [12,13], demonstrating that PEO chains migrate to the interface under aqueous conditions, significantly reducing protein adsorption. Additionally, we have examined the generation of reactive Si-H groups on PDMS and their subsequent hydrosilylation with allyl terminated PEO [14]. The resultant surfaces were found to have a high density of PEO, which led to significant reductions in the adsorption of plasma and tear proteins.

Alternatively, modification of silicone elastomers with various biomolecules has been employed to direct both biochemical and cellular responses. For example, heparin has been immobilized onto silicone surfaces in order to impart anticoagulant properties [15]. Cell adhesion peptides have also been incorporated onto silicone surfaces in order to improve cell adhesion and proliferation [16,17]. However, there is growing evidence that cell receptor interactions with surface-conjugated ligands are influenced not only by surface ligand density but also ligand spatial freedom and micro distribution [18,19]. Therefore, tethering biological ligands via a flexible spacer is believed to improve biological response [20]. By using a heterobifunctional PEO containing an allyl terminus for hydrosilylation and an NSC terminus (allyl-PEO-NSC) for reaction with the amine groups of biological molecules, generic bioaffinity PDMS surfaces have been generated [21]. These surfaces have been modified at high density with such biological molecules as cell adhesion peptides, heparin, and proteins including EGF, albumin, and lysozyme. However, separate synthesis methods are necessary for the surfaces and the allyl-PEO-

NSC. Preparation and purification of the allyl-PEO-NSC is a laborious multistep process and the resulting PDMS-PEO-NSC surfaces have a limited shelf life. Herein we report a robust and facile synthesis method for generating these surfaces. Similar to our previous work, we use PEO as a linker molecule for attachment of biologically active molecules to the PDMS. However, in the current work, the immobilized PEO is post functionalized with the NHS groups which can then be modified with various biologically active groups. We use cell adhesion peptides as model functional groups in order to exploit cell integrin interactions with biomolecules to generate promising PDMS surfaces.

Materials and Methods

Reagents and physical methods

Poly(ethylene glycol) monoallylether (MW 550Da) (allyl-PEO-OH) was received from Clariant Inc. (Montreal, Canada). Karstedt's Pt catalyst (2–3 wt% Pt concentration in xylene, $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$), trifluoromethanesulfonic acid ≥99% (*triflic acid,* CF_3SO_3H), *N,N'*-disuccinimidyl carbonate, Arg-Gly-Asp-Ser (RGDS) peptide, diethylene glycol dimethyl ether (*2-methoxyethyl ether*), and all solvents, including methanol (anhydrous), hexane, and acetonitrile (anhydrous) were purchased from Aldrich Chemical Co. (Oakville ON) and used as received. Silicone Elastomer Kit (Sylgard 184 curing agent and silicone elastomer base) and DC1107 ($(MeHSiO)_n$) were purchased from Dow Corning (Midland, MI). Fibrinogen was received from Calbiochem (La Jolla, CA) and was radiolabeled with $Na^{125}I$ (ICN, Irvine, CA).

Advancing and receding sessile drop contact angles were measured on unmodified, PEO grafted surfaces and peptide modified surfaces using a Ramé Hart NRL CA goniometer (Mountain Lakes, NJ). Milli-Q water (18 MΩ/cm) was used with a drop volume of approximately 10 μL.

XPS was performed at Surface Interface Ontario, University of Toronto. The samples were analyzed using a Leybold Max 200 X-ray photoelectron spectrometer with a MgK non-monochromatic X-ray source. The spot size used in all cases was 2×4 mm. Survey scans were performed from 0 to 10,000 eV. Both low-resolution and C1s high-resolution analyses, with a scan width of 20 eV, were performed. The raw data were analyzed and quantified using the software Specslab (specs Gmbh, Berlin).

Attenuated Total Reflection Fourier Transform IR Spectroscopy (ATR-FTIR) measurements were carried out on a Bruker TENSOR (Bruker Instruments, Billerica, MA), Fourier transform infrared spectrometer.

Preparation of PDMS disks

PDMS was prepared according to directions provided by the manufacturer. Briefly, Sylgard 184 elastomer base was mixed thoroughly with its curing agent (10:1 (w/w)) and poured into a polystyrene dish. The mixture was degassed under vacuum for 2 hours and left to cure for a minimum of 2 days at room temperature. After curing, the PDMS film was cut into disks 8 mm in diameter and approximately 0.5 mm thick.

Si-H surface functionalization of PDMS disks

In order to create reactive sites for subsequent attachment of PEO, the surfaces were modified by incorporating an Si–H functional group on the PDMS surface as previously described [22]. The PDMS disks were placed in a solution of $(MeHSiO)_n$ (DC1107) and methanol (anhydrous) (DC1107:methanol, 3:5 (v/v)) containing triflic acid as catalyst (2% triflic acid in methanol (%v)) and the mixture shaken 30 minutes. The PDMS disks were then removed and washed thoroughly with methanol (anhydrous) and hexane. The disks were subsequently dried under N_2 and placed under vacuum for 8 hours at room temperature.

Surface attachment of PEO to PDMS by hydrosilylation

As previously described [14], DC1107 (Si-H) modified PDMS disks were placed in a solution of diethylene glycol dimethyl ether and poly(ethylene oxide) monoallylether (allyl-PEO-OH, MW 550), (diethylene glycol dimethyl ether:poly(ethylene oxide) monoallylether, 3:1 (v/v)). Karstedt's Pt-catalyst (platinum-divinyltetramethyldisiloxane complex) (15μL) was added to the reaction solution and the mixture shaken for 2 hours at room temperature. The PEO-OH modified PDMS disks were subsequently washed thoroughly with acetone, dried under N_2, and placed under vacuum for 12 hours at room temperature.

Addition of *N*-succinimidyl carbonate (NSC) to surface grafted PEO

PDMS disks containing surface grafted PEO-OH were placed in a solution of acetonitrile (1mL), triethylamine (0.1mL), and *N,N'*-disuccinimidyl carbonate (0.2g, 0.781mmol), the reaction vial purged with nitrogen, and the mixture shaken for 6 hours. The PEO-NSC modified PDMS disks were then washed with acetonitrile and diethylene glycol dimethyl ether, dried under N_2 and placed under vacuum for 12 hours at room temperature. The NSC functionalization scheme is shown in Scheme 1.

Attachment of cell adhesion peptide

PEO-NSC modified PDMS surfaces were soaked in phosphate buffered saline (PBS, pH 7.4) containing 25μg/mL Arg-Gly-Asp-Ser (RGDS) peptide for 2 hours to generate peptide modified disks as shown in Scheme 2. The PDMS-PEO-peptide disks were then rinsed thoroughly with buffer and dried under N_2.

^{125}I Radiolabeling of Fibrinogen

Fibrinogen was first dissolved in phosphate buffered saline (PBS, pH 7.4) at a concentration of 10mg/mL. Fibrinogen was then labeled with ^{125}I using the iodine monochloride (ICl) method [23] in which fibrinogen was reacted in a 1:4 molar ratio with ICl reagent and 5 μL of $Na^{125}I$ (ICN Pharmaceuticals, Irvine, CA). Free iodide was removed by passing the labeled protein solution through an AG 1-X4 column (Bio-Rad Laboratories, Hercules, CA, USA). Residual free iodide in the protein solution recovered from the column was determined by precipitation of the protein using trichloroacetic acid

(TCA). Protein solutions containing less than 3% unbound ^{125}I were used for subsequent protein adsorption experiments to sample surfaces.

Fibrinogen Adsorption to Modified PDMS Surfaces

To assess protein adsorption characteristics of the modified surfaces, fibrinogen adsorption experiments were performed. ^{125}I labeled fibrinogen was mixed with unlabeled fibrinogen (1:20, labeled:unlabeled) at a total concentration of 1 mg/mL and serially diluted with PBS buffer. Modified PDMS surfaces were incubated with 250 μL fibrinogen solutions of various concentrations for 3 h at room temperature (~22°C) in the wells of 96 well flat-bottom multi-well plates (Beckton Dickson Labware, Franklin Lakes, NJ). The surfaces were then rinsed three times for 10 min each in PBS, wicked onto filter paper, and transferred to vials for determination of radioactivity using a gamma counter (Wallac 1480 Wizard 3" Automatic, Perkin-Elmer Life Sciences, Turku, Finland). Radioactivity was converted to amounts of adsorbed protein by comparison to radioactive fibrinogen solutions of known concentration as determined using a UV spectrophotometer (Beckman Coulter, Fullerton, CA).

Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC, Manassas, VA) were cultured in F-12K cell culture medium containing 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich, Oakville, ON), and 10% fetal bovine serum (Invitrogen,

Burlington, ON) in 75 cm^2 vented flasks (BD Bioscience, Franklin Lakes, NJ). Cells were incubated at 37°C in 5% CO_2. Medium was replaced three times per week and cells were passaged at near confluence. Cells were passaged a maximum of four times prior to seeding on modified surfaces.

Cell Growth on PEO-RGDS modified PDMS

To assess whether the peptides on the surface were active, growth of human umbilical vein endothelial cells on the surfaces was examined. HUVECs were seeded on PDMS, PDMS-PEO, and PDMS-PEO-RGDS (25 μg/mL) surfaces at a density of 45 000 cells/cm^2. All surfaces were sterilized prior to seeding by soaking in 70% ethanol under aseptic conditions for 15 min and rinsed thoroughly using sterile PBS (3 x 250 uL). The seeded surfaces were cultured in well plates containing 250 μL of culture medium and incubated at 37°C in 5% CO_2. Culture medium was replaced every 2 days over a period of 10 days. Seeded surfaces were observed under a light microscope at 100X magnification.

Results

Surface characterization

ATR-FTIR

The ATR-FTIR spectra of PEO-OH modified PDMS disks, PEO-NSC modified PDMS disks, as well as the Si-H modified and unmodified PDMS disks are shown in Figure 1. As expected, the spectra of both unmodified and modified surfaces are dominated by the

distinguishing peaks of the silicone elastomer substrate. However, features characteristic of each modification are clearly present. The spectra of surfaces modified with $(MeHSiO)_n$ contain a prominent peak at 2166 cm^{-1} representing the stretching vibrations attributable to the Si–H functional group. Following hydrosilylation, the Si-H peak had completely disappeared and there was an appearance of a broader CH_2 stretching vibration around 2870 cm^{-1} corresponding to the CH_2–O group on the PEO-OH and PEO-NSC modified surfaces. A broad peak between 3700 and 3250 cm^{-1} is visible on the PEO-OH surfaces corresponding to the terminal OH group. Following the addition of *N,N'*-disuccinimidyl carbonate to the PEO-OH modified surface, a sharp peak at 1742 cm^{-1} is visible and is attributable to the C=O stretch of the NSC group now present at the terminus of the PEO chain. These results clearly suggest that the PDMS disks have been successfully modified by the PEO-NSC.

Water contact angles

Advancing and receding sessile drop water contact angles measured on the control PDMS, and PEO-OH modified PDMS are summarized in Figure 2. Unmodified PDMS showed characteristically high advancing and receding water contact angles (~107°) as expected. Contact angle hysteresis was generally small on the control surface in comparison to the PEO modified surfaces, likely the result of the smoothness and homogeneity of the silicone rubber in comparison to the relative inherent heterogeneous nature of the modified surfaces.

Following PEO modification, significant decreases in the contact angles were observed as expected, with advancing angles decreasing from 107±2° to 50±5°, and receding, from 95±2° to 32±6°, clearly indicating a significant increase in surface hydrophilicity upon surface grafting of PEO relative to unmodified PDMS. Attachment of the RGDS peptide to the PEO modified surfaces resulted in an increase in advancing and receding water contact angles to 73±6° and 57±4° respectively.

X-ray photoelectron spectroscopy

The elemental compositions of the surfaces, as measured by XPS, are summarized in Table 1. Following modification with PEO-OH, the C1 s content increased and the Si 2p content decreased at all takeoff angles, as expected. Successful grafting of the RGDS peptide is confirmed by the appearance of elemental nitrogen at a 90° takeoff angle due to the amino groups of the peptide amino acid sequence. High-resolution C1s spectra for the unmodified and modified PDMS surfaces at a 90° takeoff angle are summarized in Figure 3. The peak at a binding energy of 284.4 eV corresponds to the methyl carbons in PDMS. The small peaks at 286.3 and 288 eV are likely attributable to contamination. The peak at 286.5 eV visible in the PEO and PEO-RGDS modified PDMS spectra corresponds to carbon in the surface grafted PEO chain.

Protein Adsorption

Fibrinogen adsorption to unmodified and modified PDMS disks is summarized in Figure 4. Unmodified PDMS surfaces showed the highest level of fibrinogen adsorption at all

fibrinogen solution concentrations with a maximum adsorption of 1μg/cm^2 occurring at a fibrinogen solution concentration of 1 mg/mL. Fibrinogen adsorption was significantly lower ($p<0.05$) on both PEO and PEO-RGDS modified surfaces for all fibrinogen solution concentrations when compared to unmodified PDMS surfaces. Surfaces modified with PEO-RGDS demonstrated significantly higher ($p<0.05$) fibrinogen adsorption at all concentrations when compared to PEO-OH modified surfaces, excluding the PEO-RGDS (25 μg/mL peptide solution) surfaces exposed to a 1 mg/mL fibrinogen solution. Maximum average reductions in fibrinogen adsorption of 88% and 66% were achieved at a fibrinogen solution concentration of 0.1 mg/mL for PEO and PEO-RGDS modified surfaces demonstrating the effectiveness of the PEO modification at reducing non-specific protein adsorption.

Cell Culture on Modified PDMS Surfaces

HUVECS were seeded and cultured on both RGDS (generated in 25 ug/mL peptide reaction solution) modified and unmodified PDMS surfaces as shown in Figure 5. Despite the presence of serum in the culture medium, cells seeded on unmodified PDMS surfaces appeared to diminish in number over a period of ten days. At two days of culture, limited numbers of cells appeared to be adherent to the surface when compared to RGDS modified surfaces. At 6 days of culture, there was a decreased number of cells on the PDMS surfaces and those remaining appeared to have formed small aggregates. After ten days of culture, the remnants of cellular materials appeared to have aggregated and few viable cells appear to be present. In comparison, cells seeded on the PEO-RGDS

modified PDMS surfaces appeared to increase in number over ten days of culture, with the presence of a nearly confluent layer of cells apparent at the location of seeding after 10 days. On tissue culture polystyrene under identical culture conditions, confluence is achieved after approximately 7 days of culture.

Discussion

PEO is widely exploited for its resistance to protein adsorption in biological systems. In this work, PEO was used as a generic linker molecule for attachment of cell adhesion peptides to a silicone elastomer. Functionalization of the PEO with NSC allows for subsequent surface attachment of a variety of biomolecules via reaction with amine groups. Surface modification was achieved by means of a straightforward synthetic procedure. Surfaces were initially functionalized with Si–H groups by means of an acid-catalyzed reaction with $(MeHSiO)_n$ in methanol. The success of this reaction is evidenced by the high intensity of the Si–H absorption peak at 2166 cm^{-1} in the ATR-FTIR spectra of the PDMS elastomers modified by hydrosilane. PEO was grafted onto the silicone elastomer surfaces using a platinum-catalyzed hydrosilylation reaction between the surface Si-H groups and allyl group of the PEO chain. The absence of the Si-H band at 2166 cm^{-1} in the ATR-FTIR spectra following this reaction indicates the successful consumption of the Si-H functional group during the hydrosilylation reaction and the appearance of a broad signal around 2870 cm^{-1}, demonstrates successful PEO grafting. In previous work the PEO was pre-functionalized with NSC. However, this necessitated a lengthy purification and separate surface modification and PEO functionalization steps. As the NSC group on the surface, while relatively stable, can react under atmospheric conditions, surfaces were PEO modified immediately prior to peptide functionalization. The current method is simpler, resulting in high density PEO surfaces which are functionalized in a facile procedure to generate surfaces with high reactivity to biological

molecules. Successful functionalization with the NSC is demonstrated by the appearance of a sharp peak at 1742 cm^{-1} indicating the presence of C=O of the ester group.

Surfaces modified with PEO-OH also exhibited significantly increased wettability, as indicated by a decrease in the water contact angle compared to unmodified PDMS. XPS results demonstrated a significant decrease in the Si2p peak similar to that observed previously with grafting of the pre-functionalized PEO. High resolution XPS showed a distinct peak with a binding energy of approximately 286 eV, corresponding to C–O, on all of the PEO-modified surfaces providing clear evidence that PEO was successfully grafted onto the surface. The appearance of nitrogen on the peptide modified surfaces as seen by XPS confirms the successful attachment of the RGDS peptide to the surface. While depth profiling of these samples suggests more PDMS near the surface, this is likely the result of the high vacuum environment of the XPS and not a reflection of the level of peptide modification with these surfaces. Similar results were observed in our previous study [24]. This observation may also suggest that the PEO layer is compressed upon grafting of the peptide. Furthermore, the peptide may reside more closely to the surface then does the PEO chain due to hydrophilic/hydrophobic interactions with PDMS.

Protein adsorption to unmodified and modified surfaces was determined by adsorption of ^{125}I radiolabeled fibrinogen from buffer. As expected, unmodified PDMS adsorbed the greatest quantity of fibrinogen at all solution concentrations. Samples containing surface

grafted PEO adsorbed significantly less fibrinogen with a maximum reduction in adsorption of 88% occurring at a fibrinogen solution concentration of 0.01 mg/mL. Attachment of the RGDS peptide resulted in a slight increase in protein adsorption when compared to surfaces modified with PEO alone for all fibrinogen concentrations. Peptide modified surfaces achieved a maximum reduction in protein adsorption of 66% at a fibrinogen solution concentration of 0.01 mg/mL. No significant variation in fibrinogen adsorption was observed between surfaces of varying peptide concentrations for all fibrinogen solution concentrations tested. These results suggest that the PEO linker maintains a significant portion of its protein repelling capability upon attachment of the RGDS peptide. Any increase in protein adsorption is presumably attributable to a reduction in the surface hydration layer required for protein repulsion upon conjugation with the peptide [9,25]. The large increase in chain molecular weight with the addition of the peptide may also affect the non fouling capability of the surface grafted PEO.

Bioactivity of the surface grafted peptides was observed by culturing human umbilical vein endothelial cells on the modified surfaces. Observation of cell growth under the light microscope (Figure 6) revealed that cells seeded on unmodified PDMS failed to form a confluent layer of cells by 10 days. PEO-RGDS modified surfaces however resulted in a nearly confluent layer of cells over the seeded area of the surface by 10 days of culture. This result suggests that the peptides are both present and active on the surface of the peptide modified surfaces generated by incorporation of the PEO linker as described herein.

Conclusions

Silicone elastomer surfaces were functionalized with Si–H groups by acid-catalyzed equilibration of PDMS in the presence of $(MeHSiO)_n$. PEO-OH was subsequently grafted to these surfaces using a platinum-catalyzed hydrosilylation reaction. The grafted allyl-PEO-OH was subsequently modified to generate a reactive NSC ester group for subsequent functionalization. ATR-FTIR, XPS and water contact angle results confirmed the presence of PEO on the surface of the silicone rubber. The cell adhesion peptide RGDS was grafted to PEO-NSC surfaces to improve the potential for these materials to be used in blood contacting applications. Both PEO and PEO-RGDS modified surfaces demonstrated significant reductions in fibrinogen adsorption, a maximum of 88% and 66% respectively, when compared to unmodified PDMS controls. This suggests that the PEO modified surfaces are able to reduce non-specific adsorption of proteins and that the modification with RGDS, while altering the protein repellant properties of these materials to some extent, does not eliminate the desirable effects of the PEO. Peptide modified surfaces sustained growth of endothelial cells over a ten day period thereby confirming surface grafted peptide bioactivity.

Acknowledgements

Funding support from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

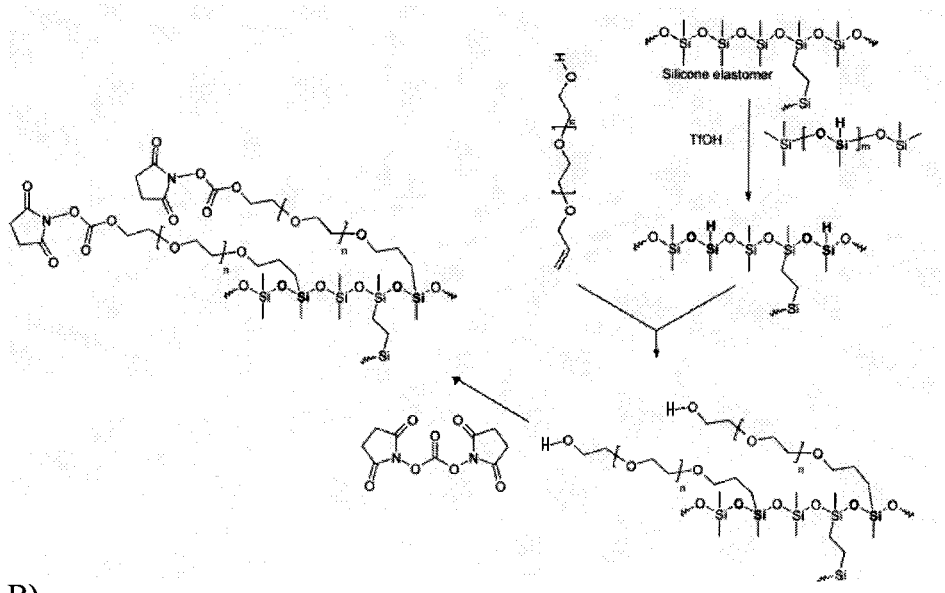
List of Figures

Figure 4. ^{125}I radiolabeled fibrinogen adsorption from PBS buffer (pH 7.4) after 3 hours of exposure (n=4). "*" indicates a significant difference ($p \leq 0.05$) in fibrinogen adsorption compared to PDMS controls and PEO-OH modified surfaces. "+" indicates a significant difference ($p \leq 0.05$) in fibrinogen adsorption compared to PDMS surfaces only. Error bars represent standard deviation.

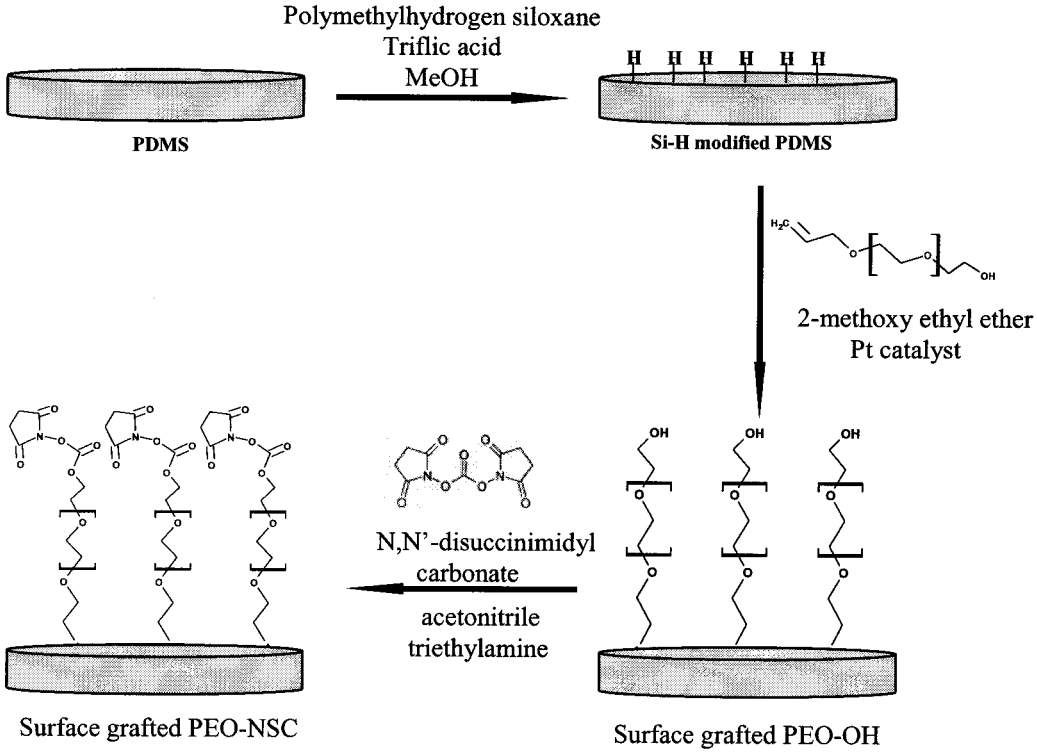
Figure 5. PDMS and PDMS-PEO-RGDS surfaces were seeded with HUVECs at an initial density of 45 000 cells/cm^2 and observed under a light microscope at 100X magnification. Images were captured after 2, 6, and 10 days of incubation.

Scheme 1

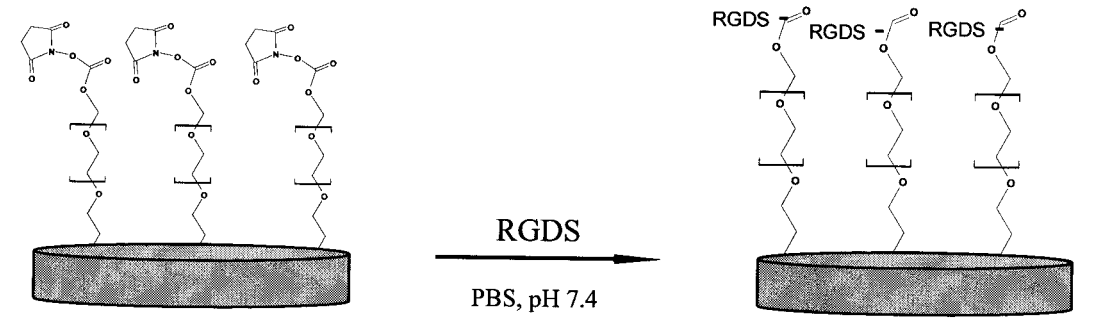
A)


Silicone elastomer
TfOH

B)


Polymethylhydrogen siloxane
Triflic acid
MeOH
PDMS
Si-H modified PDMS
2-methoxy ethyl ether
Pt catalyst
N,N'-disuccinimidyl
carbonate
acetonitrile
triethylamine
Surface grafted PEO-NSC
Surface grafted PEO-OH

Scheme 2



PEO- NSC functionalized PDMS

PEO-RGD functionalized PDMS

Figure 1

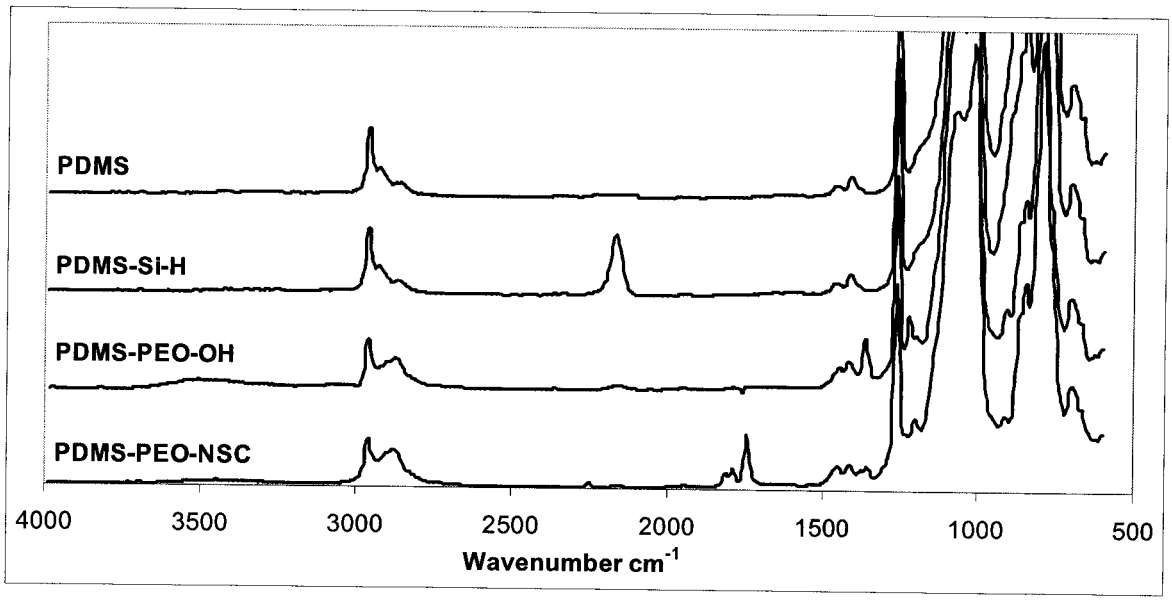


Figure 2

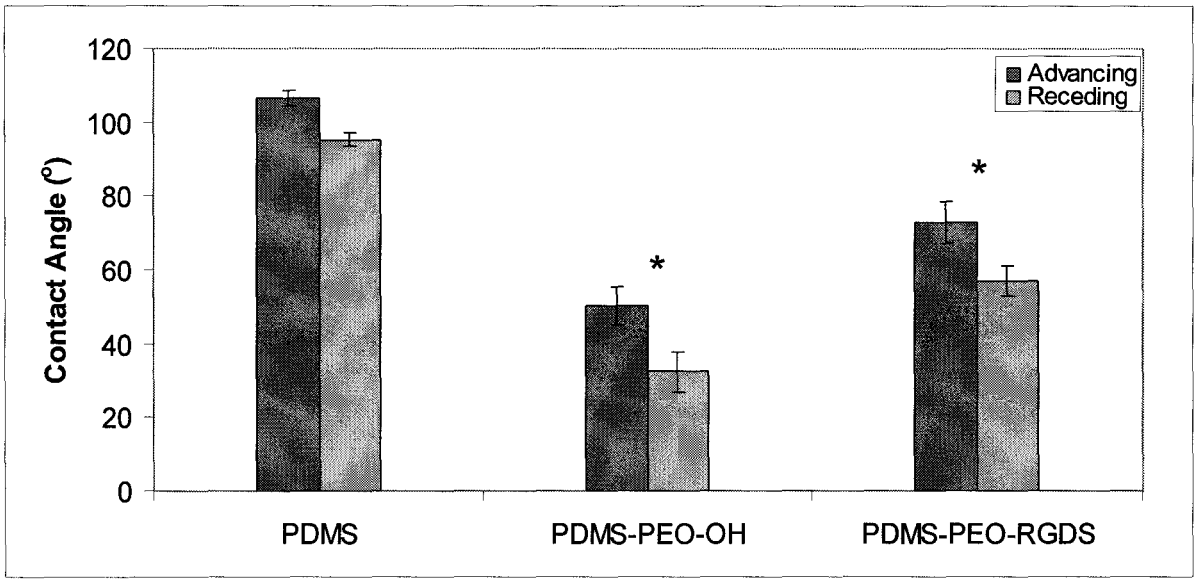


Table 1. Elemental XPS data for PDMS, PDMS-PEO-OH, and PDMS-PEO-RGDS surfaces at various takeoff angles.

	Takeoff Angle (°)											
	90				30				20			
Surface	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p
PDMS	43.5	0	27.2	29.2	45.5	0	25.6	28.8	46.2	0	27.2	26.6
PDMS-PEO-OH	59.5	0	28.1	12.4	55.8	0	27.2	17.1	56.6	0	26.2	17.2
PDMS-PEO-RGDS	51.3	0.6	26.5	21.7	47.9	0	26.1	26	50.5	0	25	24.6

Figure 3

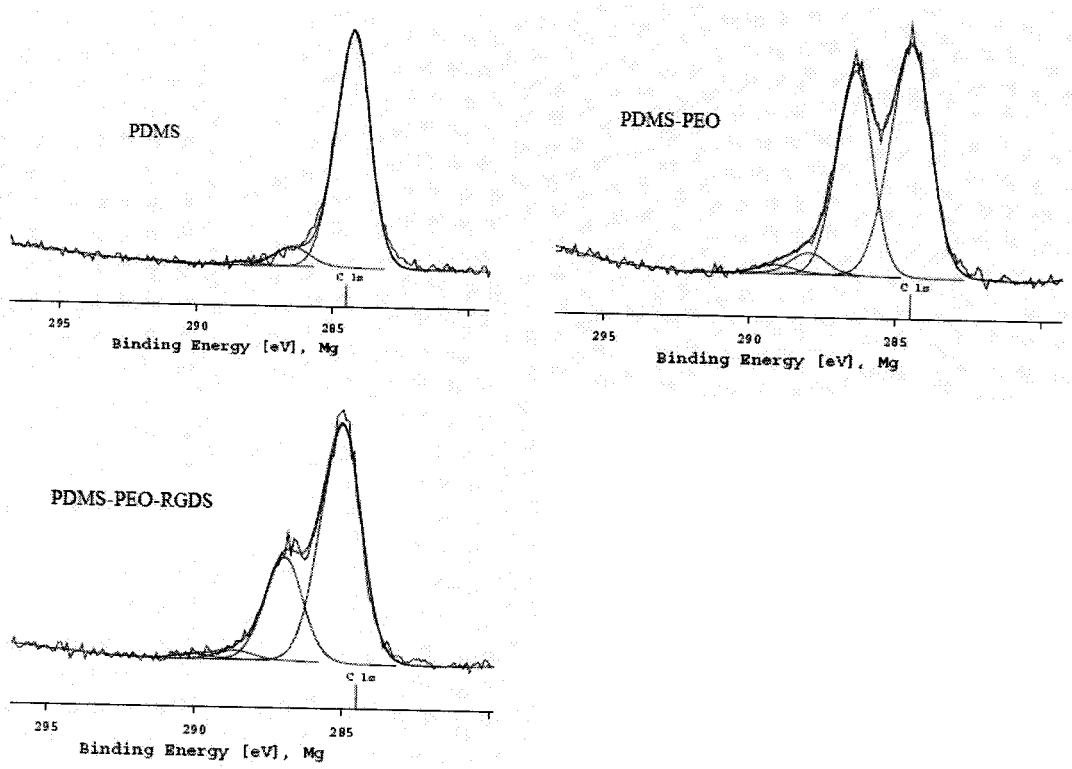

PDMS
C 1s
295
290
285
Binding Energy [eV], Mg
PDMS-PEO
C 1s
295
290
285
Binding Energy [eV], Mg
PDMS-PEO-RGDS
C 1s
295
290
285
Binding Energy [eV], Mg

Figure 4

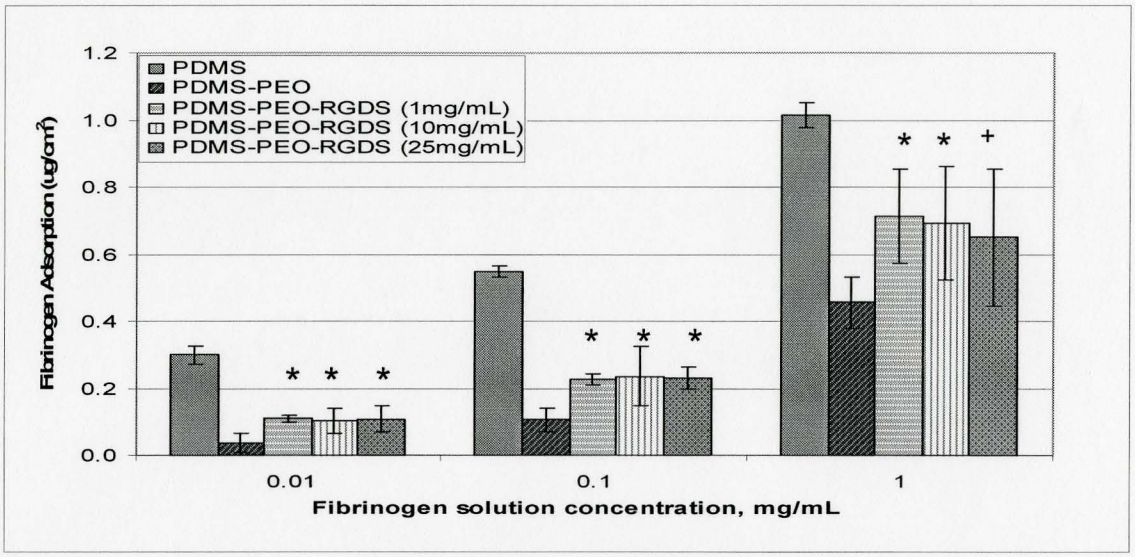
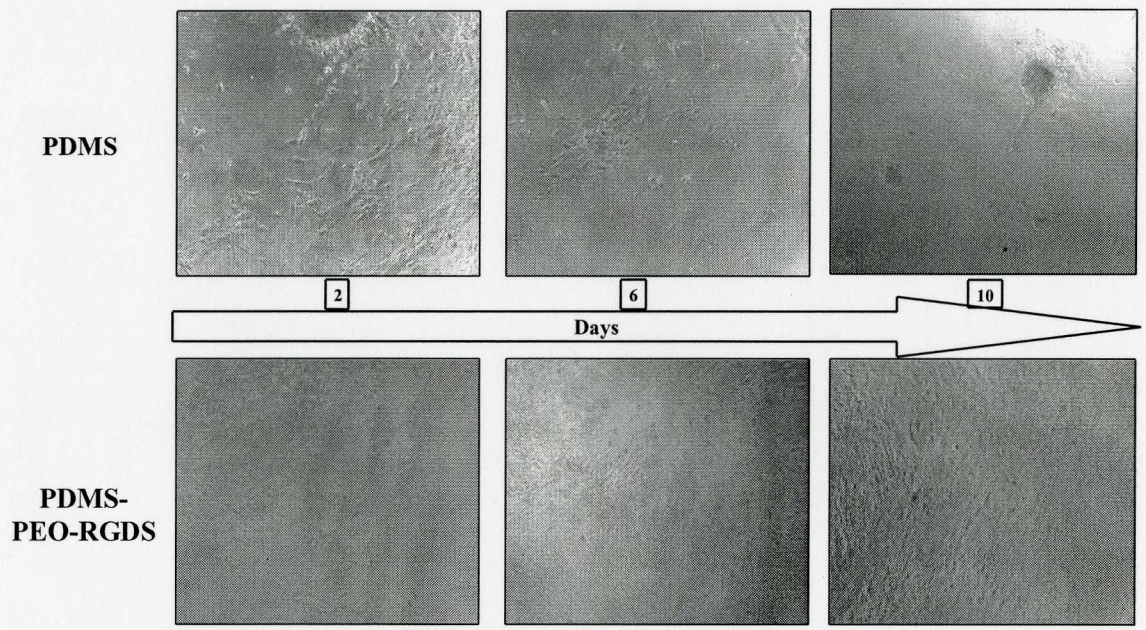


Figure 5



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7 PAPER TWO: GENERIC PDMS MODIFICATION USING A PEO LINKER FOR ATTACHMENT OF RGD PEPTIDES

Authors: A.S. Mikhail, J.J. Ranger, L. Liu, K.S. Jones, H. Sheardown, M.A. Brook

Working Hypothesis:

A PEO linker molecule can be used for attachment of cell adhesion peptides at controllable surface densities for generation of highly bioactive surfaces capable of supporting high levels of cell adhesion.

Main Scientific Contributions:

1. Demonstrated a method for generating high density peptide modified silicone elastomers as quantified by radiolabeling techniques.

2. Demonstrated that the extent of cell adhesion to PEO-peptide modified PDMS surfaces can be controlled by changing surface peptide density.

Generic PDMS Modification Using a PEO linker for Attachment of RGD Peptides

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Abstract

Cell adhesion peptides were surface grafted to polydimethylsiloxane elastomers via a bifunctional allyl-, NSC-terminated PEO linker molecule in order to improve endothelial cell adhesion. Attachment of the linker molecule to hydrosilane functionalized silicone surfaces was achieved via hydrosilylation using the allyl-terminated PEO linker. Amine-containing peptides were subsequently attached to the PEO linker via reaction with the terminal NSC group. ATR-FTIR, X-ray photoelectron spectroscopy (XPS), and water contact angles confirmed the successful attachment of the polymer linkers while XPS also confirmed the presence of surface grafted peptides. High surface grafting density of the peptides was demonstrated using radiolabeling techniques. Biological activity was demonstrated by a 5 fold increase in cell adhesion on the peptide modified surfaces when compared to unmodified PDMS control surfaces.

Keywords: PDMS, PEO, cell adhesion peptide, generic modification

Introduction

As our understanding of biological responses to foreign materials has grown, so too has the potential for creating bioactive materials capable of inducing and directing beneficial cellular processes. Poly(dimethylsiloxane) (PDMS) has many desirable properties that make it an attractive material for various biomedical applications [1]. However, its use is limited in both blood contacting and ophthalmic applications by the fact that, as with most polymers, nonspecific protein adsorption occurs following exposure to protein-containing solutions [2,3]. Non-specific adsorption of blood proteins has been widely demonstrated to mediate subsequent biological reactions including coagulation, and platelet adhesion and activation [4].

Covalent attachment of passivating polymers, such as poly(ethylene oxide) (PEO) has been demonstrated in numerous studies to reduce non-specific protein adsorption. However, methods for PEO modification of PDMS are limited due to a lack of reactive functional groups on the PDMS surface. Most techniques for such modifications involve the generation of a functional layer prior to surface grafting [5-7]. These methods can be laborious, are not highly reproducible, often result in incomplete surface coverage with the target functional molecule, and are limited by the highly surface-active nature of PDMS which tends to lead to reorientation of the polymer.

Alternatively, modification with biologically active ligands, such as cell adhesion peptides [8-10], has been employed in order to ameliorate cellular interactions. For blood contacting applications, similar modifications may target the generation of an endothelial cell layer for mimicry of the blood-lumen interfacial properties of native blood vessels. Though it is generally accepted that peptide surface density influences cell adhesion and migration [11,12], recent evidence suggests that ligand spatial freedom, as determined by spatial constraints imposed by the ligand tether molecule, is an important determinant of ligand interaction with cell integrins [13-15]. Therefore, tethering biological ligands via a highly mobile and flexible spacer is likely to improve biological response.

We have recently reported a novel modification method for silicone rubber that involves the generation of reactive Si-H groups on the PDMS surface and their subsequent hydrosilylation with allyl terminated PEO [16,17]. The resultant surfaces were found to have a high density of PEO, which led to significant reductions in the adsorption of plasma and tear proteins. By using a heterobifunctional PEO molecule, with a terminal NHS group, various biologically active functional groups could be tethered to the surface via this high density PEO layer [18]. As preparation and purification of the allyl-PEO-NSC is a laborious multistep process, a method was developed which permitted in situ generation of the PDMS-PEO-NSC surfaces (manuscript in preparation, see chapter 6). Herein we demonstrate the use of this method for generating high density cell adhesion peptide modified PDMS surfaces. Furthermore, we report on the potential of these surfaces for promoting interactions with vascular endothelial cells.

Materials and Methods

Reagents

Poly(ethylene glycol) monoallylether (MW 550Da) (allyl-PEO-OH) was received from Clariant Inc. (Montreal, Canada). Karstedt's Pt catalyst (2–3 wt% Pt concentration in xylene, $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$), trifluoromethanesulfonic acid ≥99% (triflic acid, CF_3SO_3H), *N,N'*-disuccinimidyl carbonate, Arg-Gly-Asp-Ser (RGDS) peptide, diethylene glycol dimethyl ether (2-methoxyethyl ether), trifluoroacetic acid (TFA) and all solvents, including methanol (anhydrous), hexane, toluene, and acetonitrile (anhydrous) were purchased from Aldrich Chemical Co. and used as received. Silicone Elastomer Kit (Sylgard 184 curing agent and silicone elastomer base) and DC1107 ($(MeHSiO)_n$) were purchased from Dow Corning (Midland, MI). Cell adhesion peptide (Gly-Tyr-Arg-Gly-Asp-Ser, (GYRGDS) >95%) was purchased from American Peptide (Sunnyvale, CA).

Preparation of PDMS disks

PDMS was prepared according to directions provided by the manufacturer. Briefly, Sylgard 184 elastomer base was mixed thoroughly with its curing agent (10:1 (w/w)) and poured into a polystyrene dish. The mixture was degassed under vacuum for 2 hours and left to cure for a minimum of 2 days at room temperature. After curing, the PDMS film was cut into disks 8 mm in diameter and approximately 0.5 mm thick.

Surface functionalization of PDMS

In order to create reactive sites for subsequent attachment of PEO, the surfaces were modified by incorporating an Si–H functional group on the PDMS surface as previously described [19]. The PDMS disks were placed in a solution of $(MeHSiO)_n$ (DC1107) and methanol (anhydrous) (DC1107:methanol, 3:5 (v/v)) containing triflic acid as catalyst (2% triflic acid in methanol (%v)) and the mixture was shaken for 30 minutes. The PDMS disks were then removed and washed thoroughly with anhydrous methanol and hexane. The disks were subsequently dried under N_2 and placed under vacuum for 8 hours at room temperature.

Attachment of PEO to PDMS by hydrosilylation

As previously described [16], DC1107 (Si-H) modified PDMS disks were placed in a solution of diethylene glycol dimethyl ether and poly(ethylene oxide) monoallylether (allyl-PEO-OH, MW 550), (diethylene glycol dimethyl ether:poly(ethylene oxide) monoallylether, 3:1 (v/v)). Karstedt's Pt-catalyst (platinum-divinyltetramethyldisiloxane complex) (15μL) was added to the reaction solution and the mixture shaken for 2 hours at room temperature. The PEO-OH modified PDMS disks were subsequently washed thoroughly with acetone, dried by N_2, and placed under vacuum for 12 hours at room temperature.

Addition of *N*-succinimidyl carbonate (NSC) to surface grafted PEO

PDMS disks containing surface grafted PEO-OH were placed in a solution of acetonitrile (1 mL), triethylamine (0.1 mL), and *N,N'*-disuccinimidyl carbonate (0.2 g, 0.781 mmol). The reaction vial was purged with nitrogen, and the mixture shaken for 6 hours. The resulting PEO-NSC modified PDMS disks were washed with anhydrous acetonitrile and diethylene glycol dimethyl ether, dried under N_2 and placed under vacuum for 12 hours at room temperature. The reaction scheme described above is shown in Scheme 1.

Attachment of cell adhesion peptide

PEO-NSC modified PDMS surfaces were soaked in buffer solution (pH 7.4) containing either Arg-Gly-Asp-Ser (RGDS) or ^{125}I radiolabeled Gly-Tyr-Arg-Gly-Asp-Ser (GYRGDS) at the desired reaction solution concentrations for 2 hours to generate peptide modified disks as shown in Scheme 2. The PDMS-PEO-peptide disks were then rinsed thoroughly with buffer and dried under N_2.

Radiolabeling of GYRGDS cell adhesion peptide

Cell adhesion peptide GYRGDS was used for radiolabeling experiments as it contains a tyrosine amino acid for iodination. ^{125}I radiolabeling was performed using the Iodogen method. Briefly, 10 μL of $Na^{125}I$ was added to 250 μg of the peptide, dissolved in tris buffered saline (TBS) (pH 7.4) at a concentration of 1mg/mL. The solution was placed in an IODO-GEN® coated vial and stirred for 15 minutes. Separation of the peptide and unbound ^{125}I after iodination was accomplished using a reverse phase Sep-Pak C_{18}

column (Waters, Mississauga, ON) containing a silica-based bonded phase of strong hydrophobicity. The column was pre-equilibrated with 6-10 mL of methanol followed by 6-10 mL of 0.1% trifluoroacetic acid (TFA) in TBS buffer at pH 7.4. The reaction solution was poured into the column and rinsed with approximately 15 mL of 0.1% TFA in buffer. Solution fractions were collected until it was determined that all of the free iodide had eluted. Following elution of the unbound ^{125}I, the labeled GYRGDS peptide was eluted using a solution of acetonitrile and 0.1% TFA in TBS buffer (acetonitrile:0.1% TFA in TBS, 20:80 (v:v)). Solution fractions were collected and measured for radioactivity. The peptide concentration of the most radioactive sample fractions was determined by measuring the absorbance at 280 nm; sample radioactivity was measured using a gamma-counter (Wallac 1480 Wizard 3" Automatic, Perkin-Elmer Life Sciences, Turku, Finland).

Cell Culture and Adhesion to Modified Surfaces

Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC, Manassas, VA) were cultured in F-12K cell culture medium containing 2mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich, Oakville, ON), and 10% fetal bovine serum (Invitrogen, Burlington, ON) in 75 cm^2 vented flasks (BD Bioscience, Franklin Lakes, NJ). Cells were incubated at 37°C in 5% CO_2. Medium was replaced three times per week and cells were passaged at near confluence to a maximum of four passages prior to seeding on modified surfaces. To assess whether the peptides on the surface were active, adhesion of

human umbilical vein endothelial cells was examined. HUVECs were seeded on the surfaces at a density of 25 000 cells/cm^2 in medium. All surfaces were sterilized prior to seeding by soaking in 70% ethanol under aseptic conditions for 15 min and rinsed thoroughly using sterile PBS (3 x 250 uL). The seeded surfaces were incubated at 37°C for two hours to allow for cell adhesion at which time 250 μL of culture medium was added to each well. After 6 hours, the culture medium was carefully aspirated and the seeded surfaces were rinsed gently with sterile PBS (3 x 250 μL). Cell number was determined fluorometrically using the CyQUANT ® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to manufacturer instructions,.

Results

Surface characterization

ATR-FTIR

The ATR-FTIR spectra of PEO-OH modified PDMS disks, PEO-NSC modified PDMS disks, as well as the Si-H modified and unmodified PDMS disks are shown in Figure 1. As expected, the spectra of both unmodified and modified surfaces are dominated by the distinguishing peaks of the silicone elastomer substrate. However, features characteristic of the different functional groups are clearly present. The spectra of surfaces modified with $(MeHSiO)_n$ contain a prominent peak at 2166 cm^{-1} representing the stretching vibrations attributable to the Si–H functional group. Following hydrosilylation, the Si-H peak has completely disappeared and there is an appearance of a broader CH_2 stretching vibration around 2870 cm^{-1} corresponding to the CH_2–O group on the PEO-OH and

PEO-NSC modified surfaces. A broad peak between 3700 and 3250 cm^{-1} is visible on the PEO-OH surfaces corresponding to the terminal OH group. Following the addition of *N,N'*-disuccinimidyl carbonate to the PEO-OH modified surface, a sharp peak at 1742 cm^{-1} is visible and is attributable to the C=O stretch indicative of the presence of the NSC group at the terminus of the surface tethered PEO chain. These results clearly suggest that PEO has been successfully grafted to the PDMS disks and that the terminal –OH groups have been successfully modified to NSC groups.

Water contact angles

Advancing and receding sessile drop water contact angles measured on unmodified PDMS, and PEO-OH and PEO-RGDS modified surface are summarized in Figure 2. Unmodified PDMS showed characteristically high advancing and receding water contact angles (~107°) as expected. Contact angle hysteresis was generally small on the control surface in comparison to the PEO modified surfaces, likely the result of the smoothness and homogeneity of the silicone rubber in comparison to the relative inherent heterogeneous nature of the modified surfaces.

Following PEO modification, significant decreases in the contact angles were observed as expected, with advancing angles decreasing from 107±2° to 50±5°, and receding, from 95±2° to 32±6°, clearly indicating a significant increase in surface hydrophilicity upon surface grafting of PEO relative to unmodified PDMS. Attachment of the RGDS peptide

to the PEO modified surfaces resulted in an increase in advancing and receding water contact angles to 73±6° and 57±4°, respectively.

X-ray photoelectron spectroscopy

The elemental compositions of the surfaces, as measured by XPS, are summarized in Table 1. Following modification with PEO-OH, the C1s content increased and the Si 2p content decreased at all takeoff angles, as expected. Successful grafting of the RGDS peptide is confirmed by the appearance of elemental nitrogen at a 90° takeoff angle pertaining to the amino groups of the peptide amino acid sequence. High-resolution C1s spectra for the unmodified and modified PDMS surfaces at a 90° takeoff angle are summarized in Figure 3. The peak at a binding energy of 284.4 eV corresponds to the methyl carbons in PDMS. The small peaks at 286.3 and 288 eV are likely attributable to contamination. The peak at 286.5 eV visible in the PEO-OH modified PDMS spectra corresponds to carbon in the surface grafted PEO chain. The intensity of this peak is diminished on RGDS modified surfaces due to the reduced presence of C-O bonds. Though the presence of peptide C-N bonds may also contribute to this peak, the net result is a reduction in peak intensity.

^{125}I radiolabeled GYRGDS peptide surface grafting

Surface-grafted GYRGDS peptide densities are summarized in Figure 4. There is clearly a trend of increasing surface grafted peptide density with increasing peptide reaction solution concentration. The maximum density achieved was on the order of 60 pmol/cm^2

at the highest reaction solution concentration examined. Increased variability at 25 μg/mL peptide solution concentration is thought to be a result of increased steric effects that may influence peptide binding at high surface peptide densities.

Cell Adhesion

The cell adhesive nature of RGD-modified substrates was investigated by seeding HUVECs onto the sample surfaces and incubating them in medium for 6 hours. Percent cell adhesion of HUVECs is summarized in Figure 5. As expected, the lowest % cell adhesion was observed on control PEO-OH modified surfaces and PEO-NHS modified surfaces soaked in buffer containing no RGDS peptide. PDMS control surfaces appear to have slightly better cell adhesive properties than PEO-OH modified surfaces, although the difference is not statistically significant. However, on the PEO-RGDS modified surfaces, cell adhesion increased with increasing peptide solution concentration, with a maximum average cell adhesion of 87.3% on surfaces soaked in a reaction solution containing 25μg/mL of peptide.

Discussion

PEO is widely exploited for its resistance to protein adsorption in biological systems [20]. In this work, PEO was used as a generic linker molecule for attachment of cell adhesion peptides to a silicone elastomer. Functionalization of the PEO with an active NHS ester allows for subsequent surface attachment of a variety of biomolecules via reaction with amine groups. In blood contacting applications, it has been suggested that the ability to

support a vascular endothelial cell monolayer may result in improved blood compatibility. Tethering a cell adhesion molecule via the PEO linker was therefore used to generate surfaces with low non-specific adsorption of plasma proteins and high affinity for vascular endothelial cells.

Surface modification was achieved by means of a straightforward synthetic procedure through which substrates were initially functionalized with Si–H groups by means of an acid-catalyzed reaction with $(MeHSiO)_n$ in methanol. The success of this reaction is confirmed by the high intensity of the Si–H absorption peak at 2166 cm^{-1} in the ATR-FTIR spectra of the PDMS elastomers modified by hydrosilane. PEO was grafted onto the silicone elastomer surfaces using a platinum-catalyzed hydrosilylation reaction between the surface Si-H groups and allyl group of PEO. The absence of the Si-H band at 2166 cm^{-1} in the ATR-FTIR spectra following this reaction indicates the successful consumption of the Si-H functional group during the hydrosilylation reaction and the appearance of a broad signal around 2870 cm^{-1} is indicative of successful PEO grafting. Post modification with NSC permits the development of stable high density PEO surfaces which may be functionalized using a facile procedure to generate surfaces with high reactivity to biological molecules. Successful functionalization with NSC is demonstrated by the appearance of a sharp peak at 1742 cm^{-1} indicating the presence of C=O of the ester group.

Surfaces modified with PEO-OH exhibited significantly increased wettability, as indicated by a decrease in the water contact angle compared to unmodified PDMS. XPS results demonstrated a significant decrease in the Si2p peak similar to that observed previously with grafting of the pre-functionalized PEO. High resolution XPS showed a distinct peak with a binding energy of approximately 286 eV, corresponding to C–O, on all of the PEO-modified surfaces providing clear evidence that PEO was successfully grafted onto the surface. The presence of the RGDS peptide is confirmed by the detection of elemental nitrogen in the XPS spectra.

Surface peptide density was determined by radiolabeling GYRGDS peptide with ^{125}I prior to reaction with PEO-NSC modified surfaces. A clear effect of solution concentration was observed with higher levels of peptide grafting on the surfaces modified with solutions containing higher concentrations of peptide, demonstrating that the peptide grafting density could be tailored by simple alterations to the reaction conditions. A maximum average grafting density of 60.2 pmol/cm^2 was observed when the surfaces were functionalized using this method. This compares favorably with our previous results which demonstrate an EGF surface density on the order of approximately 50 pmol/cm^2 [18]. The higher ligand grafting density potential of this method may be a result of increased attachment of PEO. This may be due to the fact that the allyl-PEO-NSC previously used is difficult to separate completely from the undesired NHS impurity generated during its synthesis. The NHS impurity is capable of covalent attachment to the Si-H surface thereby reducing the availability of Si-H groups for subsequent

hydrosilylation. The current method avoids undesired consumption of surface Si-H groups, therefore allowing greater surface grafting of PEO. Initial grafting of allyl-PEO-OH may also help minimize steric effects previously associated with the bulky NSC functional group allowing for greater surface grafting potential.

Peptide grafted surfaces generated by this method are capable of supporting human umbilical vein endothelial cell adhesion. Maximum cell adhesion of 87.3% was achieved on PEO-RGDS modified surfaces with a peptide density on the order of 60 pmol/cm^2, with clear increases in adhesion with increasing peptide surface density. Cell adhesion to the modified surfaces may therefore be controlled by simply altering the reaction solution concentration to generate surfaces with different surface peptide density. In comparison, unmodified PDMS resulted in the adhesion of only 16.5% of seeded cells while surfaces modified with PEO-OH resulted in the adhesion of 10.1% of seeded cells. Serum proteins were included in the medium in order to evaluate the efficacy of the PEO linker for promotion of cell adhesion, given its protein repelling properties. We have previously reported that protein adsorption varies minimally with changes in surface peptide density using this PEO linker chemistry (manuscript in preparation, see chapter 6). Therefore, cell adhesion to these surfaces is determined largely by surface peptide density rather than the protein repelling nature of the PEO linker.

Conclusions

Cell adhesion peptide modified surfaces generated via the generic PEO linker chemistry described herein are promising surfaces for promoting vascular endothelialization of PDMS. Adhesion of human umbilical vein endothelial cells on PEO-RGDS modified substrates demonstrated a ~5 fold increase in cell retention when compared to unmodified and PEO modified controls. A maximum average peptide density of approximately 60 pmol/cm^2 was achieved on the modified surfaces resulting in cell adhesion of 87.3%. These promising in vitro results suggest that with further optimization of the PEO linker process, materials generated using this method may be used for increasing endothelialization of PDMS for use in blood contacting applications. Moreover, the straightforward method described herein for attachment of biomolecules via a generic PEO linker molecule may be used for the generation of biologically relevant surfaces for a variety of applications.

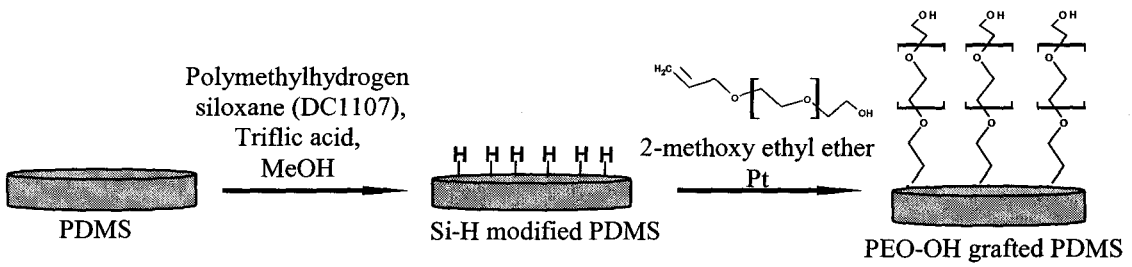
Acknowledgements

Funding support from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

List of Figures

Figure 5. Percent cell adhesion of HUVECs on RGDS peptide modified PDMS of varying surface peptide densities (n=4). "*" indicates a significant difference ($p \leq 0.05$) compared to PEO-OH controls. Error bars represent standard deviation.

Scheme 1



Scheme 2

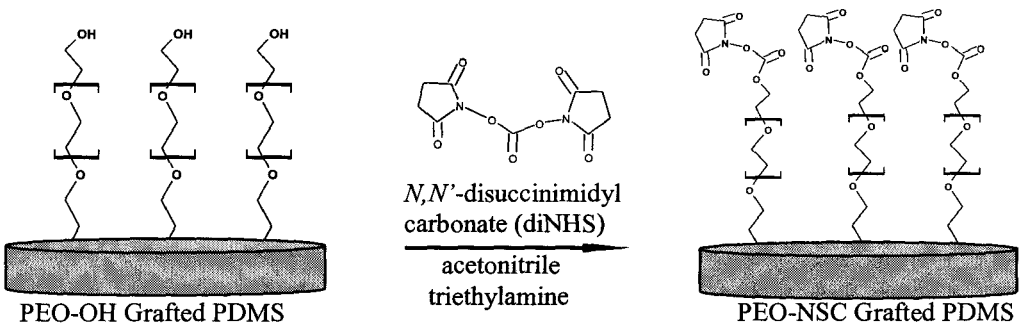


Figure 1

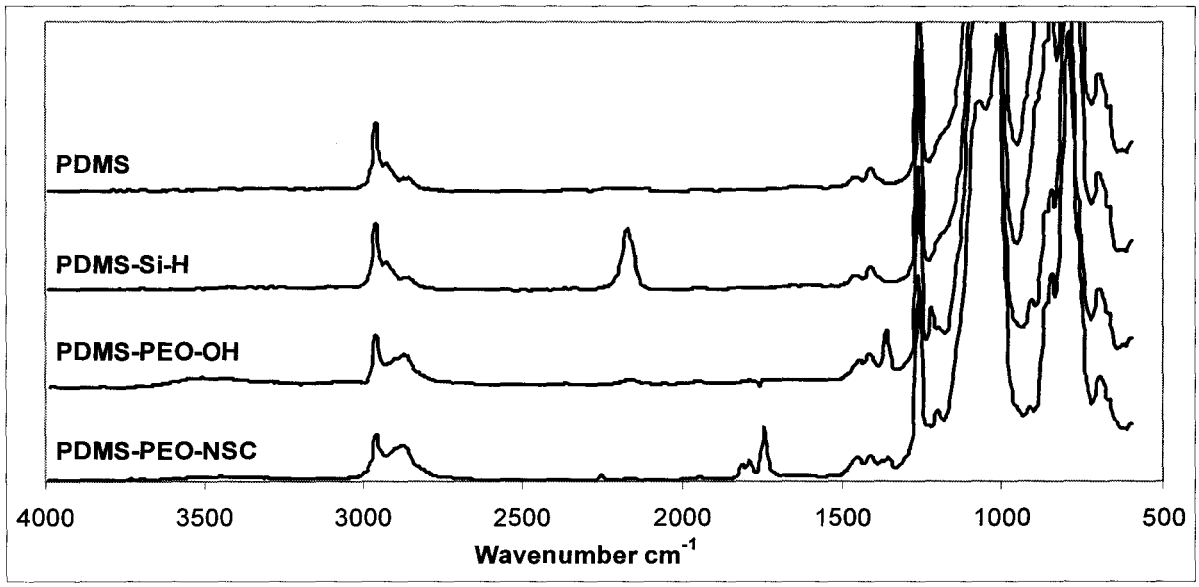


Figure 2

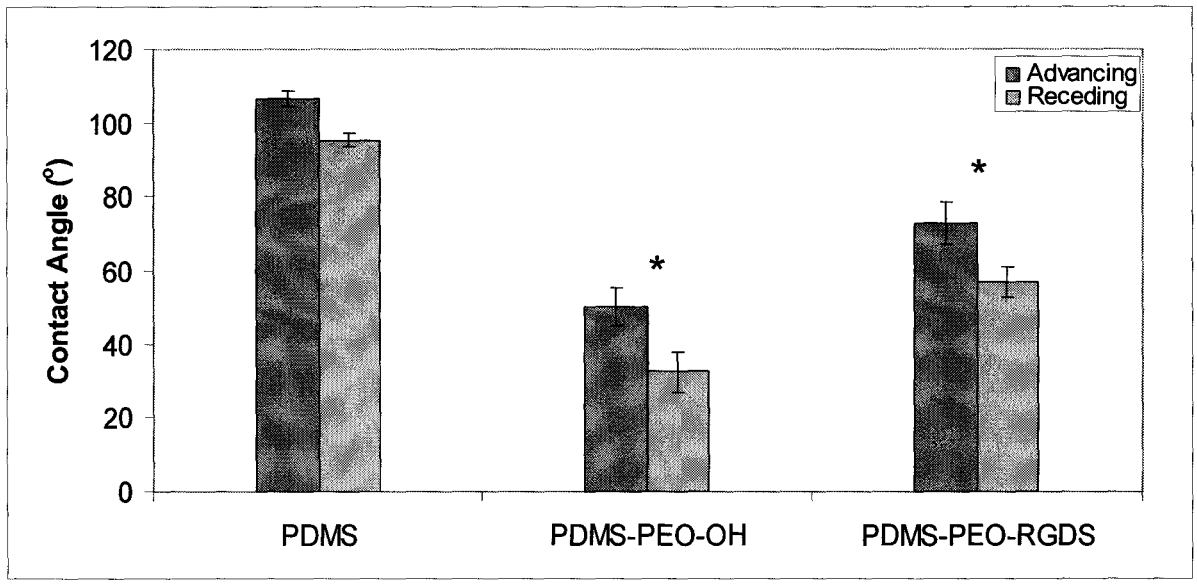


Table 1. Elemental XPS data for PDMS, PDMS-PEO-OH, and PDMS-PEO-RGDS surfaces at various takeoff angles.

	Takeoff Angle (°)											
Surface	90				30				20			
	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p
PDMS	43.5	0	27.2	29.2	45.5	0	25.6	28.8	46.2	0	27.2	26.6
PDMS-PEO-OH	59.5	0	28.1	12.4	55.8	0	27.2	17.1	56.6	0	26.2	17.2
PDMS-PEO-RGDS	51.3	0.6	26.5	21.7	47.9	0	26.1	26	50.5	0	25	24.6

Figure 3

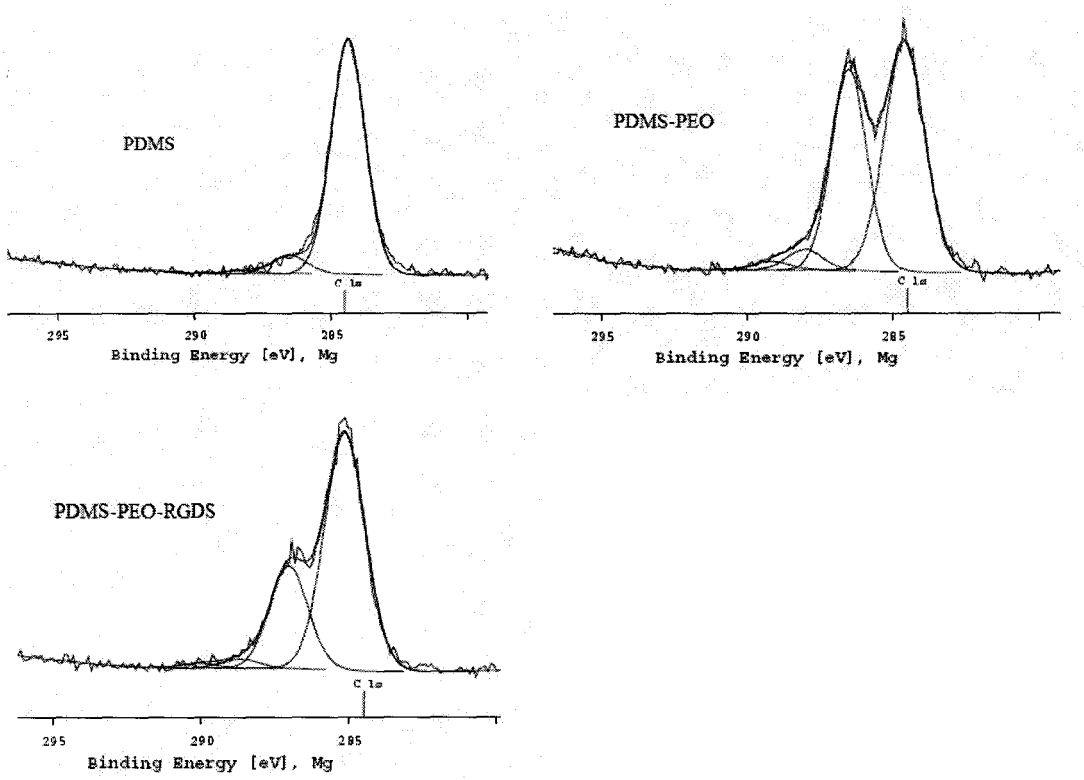


Figure 4

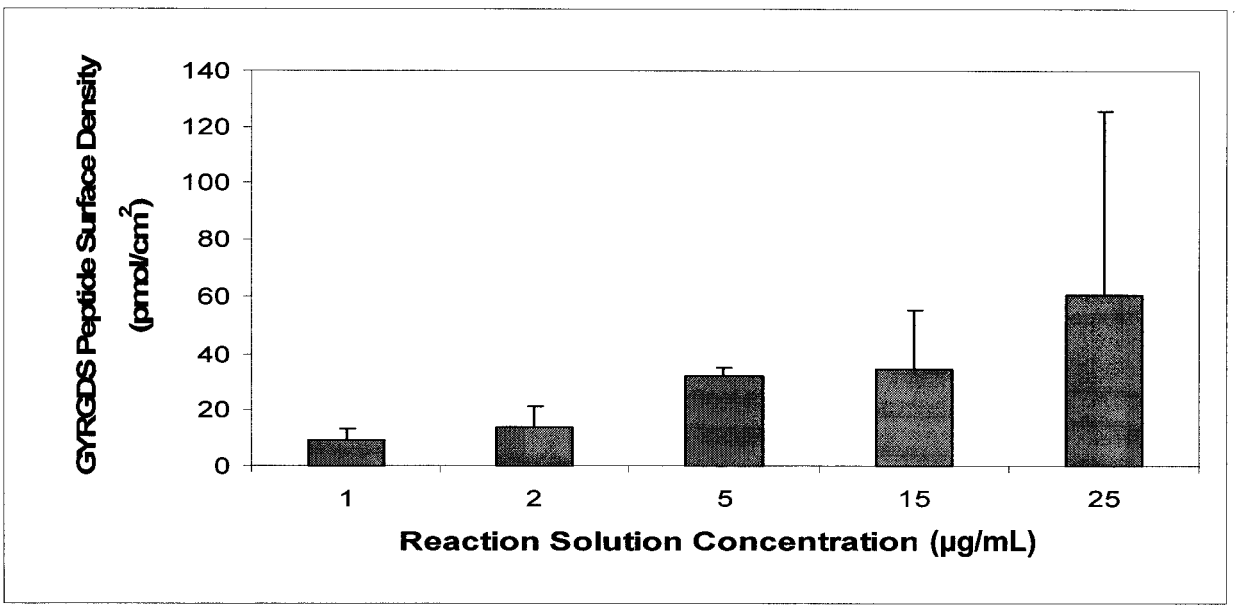
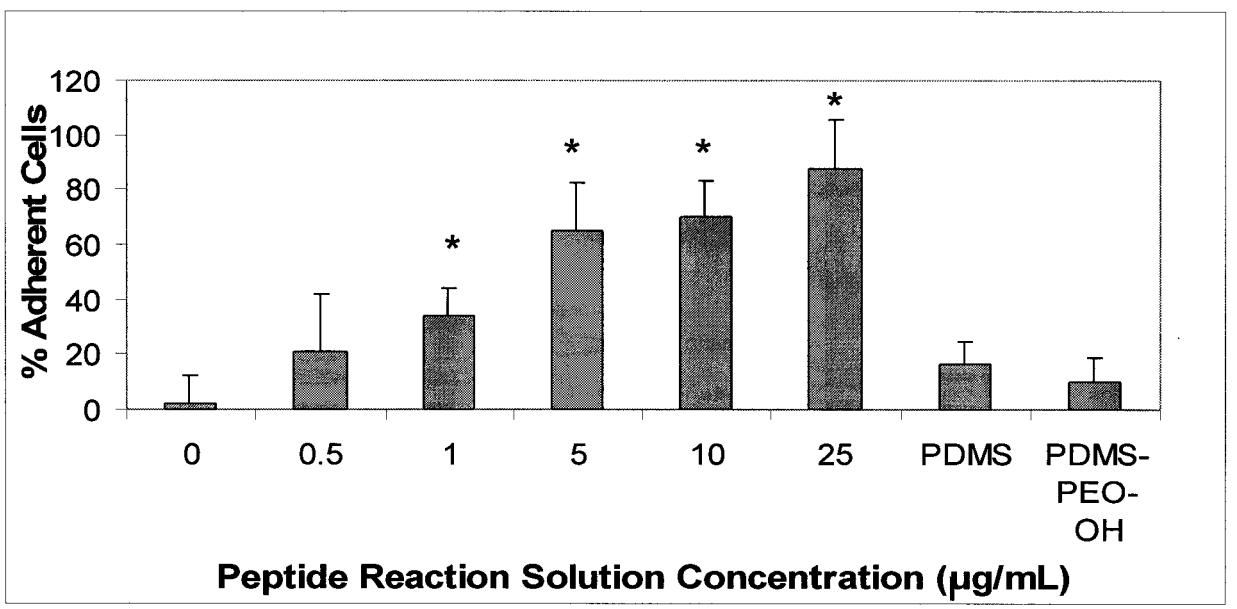


Figure 5



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8 PAPER THREE: DENDRIMER GRAFTED CELL ADHESION PEPTIDE MODIFIED PDMS

Authors: A.S. Mikhail, K.S. Jones, and H. Sheardown

Working Hypothesis:

Use of a surface grafted dendrimer linker molecule for attachment of cell adhesion peptides will result in highly functionalized surfaces with increased surface grafting capability.

Main Scientific Contributions:

1. Demonstrated a method for generating diaminobutane dendrimer modified silicone elastomers for surface grafting of amine-containing biomolecules.

2. Dendrimer modified surfaces generated by this method resulted in slightly higher peptide grafting densities compared to the peptides grafted via the PEO linker system previously described. Dendrimer surfaces were capable of high levels of peptide absorption.

3. Ligand mobility on a biomaterial surface may significantly influence ligand-cell receptor interactions to an even greater extent than surface peptide density.

Dendrimer Grafted Cell Adhesion Peptide Modified PDMS

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Abstract

Surface concentration of cell adhesion peptides is thought to play a role in the interactions between biomaterials and cells. The high density of functional groups at the periphery of dendrimers has been exploited in various applications, but has not yet been used for the development of surfaces with high functional group concentrations. Poly(dimethylsiloxane) elastomers were surface modified with both polyethylene oxide (PEO) and generation three diaminobutane dendrimers. PEO and the dendrimers were subsequently used as linker molecules for surface grafting of cell adhesion peptides and subsequent endothelial cell adhesion studies. ATR-FTIR, X-ray photoelectron spectroscopy (XPS), and water contact angle results confirmed the successful attachment of the polymer linkers and peptides. Peptide grafting density was quantified by means of ^{125}I radiolabeling. Maximum surface peptide grafting density on dendrimer modified surfaces was two fold greater than the maximum peptide grafting density achieved via the PEO linker. However, cell adhesion was significantly greater on surfaces modified with the PEO linker, presumably due to the highly flexible PEO spacer making the peptide more accessible for binding with the cell surface receptors. These results suggest that, while peptide surface density may be important, optimizing surface density may not be sufficient for improving biological interactions.

Keywords: PEO, dendrimer, PDMS, cell adhesion

Introduction

Cardiovascular disease (CVD) is the number one cause of premature mortality in the Western World [1]. Of the various cardiovascular related illnesses, the narrowing of the coronary arteries due to the deposition of plaque and formation of atherosclerotic lesions, known as coronary artery disease (CAD), accounts for greater than half of all CVD deaths [2]. Current treatment consists of coronary angioplasty or peripheral artery bypass grafting using autologous vessels including the mammary artery and saphenous vein. However, the supply of native vessels from one individual is often insufficient for multiple bypass or repeat procedures. Furthermore, autologous grafts are prone to failure due to a variety of adverse reactions associated with the graft procedure. Therefore, other sources for arterial replacements are in great demand.

The use of currently available biomaterials for small diameter vascular graft applications has been problematic due to their inherent thrombogenicity. In order to overcome this problem, much attention has been given to generating a confluent endothelial cell layer on synthetic scaffolds with the intention of mimicking the blood-lumen interfacial properties of native blood vessels. In order to ameliorate cell-biomaterial interactions, bioactive factors normally located in the extracellular matrix (ECM), including cell adhesion peptides [3-5], have been incorporated into materials to optimize cell adhesion and spreading. Peptide surface density has been widely shown and is well known to influence the degree of cell adhesion and migration on a biomaterial surface [6,7]. Recently there has been evidence that ligand spatial freedom, as determined by the spatial

constraints imposed by the ligand tether molecule, is also an important determinant of ligand bioactivity due to its effect on cell integrin interactions [8-10,28]. Thus, it is desirable to generate biomaterials with a high capacity for controllable attachment of ligands via a linker molecule conducive to increasing bioactivity.

PEO is a water-soluble, nontoxic, and non-immunogenic polymer that is highly mobile in aqueous solution. It has been widely shown that PEO, when present on a biomaterial surface, leads to a reduction in nonspecific protein adsorption [11-13]. We have previously demonstrated that attachment of cell adhesion peptides via a generic PEO linker reduces non specific protein adsorption while maintaining high levels of cell adhesion (manuscript in preparation, see chapter 6). However, in order to increase peptide surface density, alternative, non-linear polymeric structures may also be employed for attachment of cell adhesion peptides to a biomaterial surface. For example, comb [14] and star [7,15] polymer conformations have been explored as a means of controlling the distribution and density of surface bound peptides.

In the current work, we examined the use of dendrimers as novel structures for increasing the surface capacity of a biomaterial for cell adhesion peptides. Dendrimers are highly branched polymers comprised of many polymer chains and terminal functional groups making them ideal structures for surface functionalization of biomaterials or release of small proteins. Such polymeric structures have been previously employed as drug delivery vehicles [16-19], imaging agents [20-22], and carriers for gene transfection

[23,24]. We hypothesized that surface grafting of cell adhesion peptides via a diaminobutane dendrimer linker to poly(dimethylsiloxane) (PDMS) would result in increased surface peptide density when compared to peptides grafted via a linear PEO linker. Herein, we report the effect of cell adhesion peptide surface density and linker molecule structure on endothelial cell adhesion for both dendrimer and PEO modified surfaces.

2 Materials and Methods

2.1 Reagents and physical methods

Poly(ethylene glycol) monoallylether (MW 550 Da) (allyl-PEO-OH) was provided by Clariant Corporation (Markham ON, Canada). Karstedt's Pt catalyst (2–3 wt% Pt concentration in xylene, $[(Pt)_2(H_2C{=}CH\text{-}SiMe_2OSiMe_2CH{=}CH_2)_3]$), trifluoromethanesulfonic acid ≥99% (triflic acid, CF_3SO_3H), *N,N'*-disuccinimidyl carbonate, Arg-Gly-Asp-Ser (RGDS) peptide, diethylene glycol dimethyl ether (2-methoxyethyl ether), diaminobutane dendrimer (generation 3) and all solvents, including trifluoroacetic acid (TFA), methanol (anhydrous), hexane, toluene, and acetonitrile (anhydrous) were purchased from Sigma-Aldrich Chemical Co. and used as received. Silicone Elastomer Kit (Sylgard 184 curing agent and silicone elastomer base) and DC1107 ($(MeHSiO)_n$) were purchased from Dow Corning (Midland, MI). Cell adhesion peptide (Gly-Tyr-Arg-Gly-Asp-Ser, (GYRGDS) >95%) was purchased from American Peptide (Sunnyvale, CA). Scrambled peptide Arg-Asp-Gly-Ser (RDGS) was purchased from Sigma-Aldrich, Genosys.

Advancing and receding sessile drop contact angles were measured on the unmodified, PEO grafted, dendrimer grafted and peptide modified surfaces using a Ramé Hart NRL CA goniometer (Mountain Lakes, NJ). Milli-Q water (18 MΩ/cm) was used with a drop volume of approximately 10 μL.

XPS was performed at Surface Interface Ontario (Toronto ON). The samples were analyzed using a Leybold Max 200 X-ray photoelectron spectrometer with a MgK non-monochromatic X-ray source. The spot size used in all cases was 2×4 mm. Survey scans were performed from 0 to 10,000 eV. Both low-resolution and C1s high-resolution analyses, with a scan width of 20 eV, were performed. The raw data were analyzed and quantified using the software Specslab (specs Gmbh, Berlin).

Attenuated Total Reflection Fourier Transform IR Spectroscopy (ATR-FTIR) measurements were carried out on a Bruker TENSOR (Bruker Instruments, Billerica, MA), Fourier transform infrared spectrometer.

^{125}I radiolabeling of GYRGDS peptide was performed using $Na^{125}I$ (ICN Pharmaceuticals, Irvine, CA). Samples were labeled using the IODO-GEN® method. Removal of residual free iodide was accomplished using a reverse phase Sep-Pak C_{18} column (Waters, Mississauga, ON) containing a silica-based bonded phase of strong

hydrophobicity. Sample radioactivity was measured using a gamma-counter (Wallac 1480 Wizard 3" Automatic, Perkin-Elmer Life Sciences, Turku, Finland).

2.2 Preparation of PDMS disks

Sylgard 184 elastomer base was mixed thoroughly with its curing agent (10:1 (w/w)) and poured into a polystyrene dish. The polymer film was degassed under vacuum for 2 h and left to cure for a minimum of 2 days at room temperature. After curing, the PDMS film was cut into disks 8mm in diameter and approximately 0.5mm thick.

2.3 Surface functionalization of PDMS disks

In order to create reactive sites for subsequent attachment of PEO, the surfaces were modified by incorporating an Si–H functional group on the PDMS surface as previously described [25]. PDMS disks were placed in a solution containing $(MeHSiO)_n$ (DC1107) and methanol (anhydrous) (DC1107:methanol, 3:5 (v/v)). Triflic acid catalyst was added to the reaction solution containing the disks (2% triflic acid in methanol (%v)) and the vial was shaken vigorously for 30 minutes at 250 RPM. The PDMS disks were then removed and washed thoroughly with methanol (anhydrous) and hexane. The disks were subsequently dried under N_2 and placed under vacuum for 8 h at room temperature.

2.4 Preparation of PEO-grafted PDMS disks

Si-H modified PDMS disks were placed in a 2 mL solution of diethylene glycol dimethyl ether and poly(ethylene glycol) monoallylether (MW 550), (diethylene glycol dimethyl ether:poly(ethylene glycol) monoallylether, 1:1 (v/v)). Karstedt's Pt-catalyst (platinum-divinyltetramethyldisiloxane complex) (15μL) was added to the reaction solution and the mixture was shaken for 2 h at 150 RPM at room temperature. Attachment of vinyl terminated PEO to the Si-H functionalized PDMS surface was via a hydrosilylation reaction. Selectivity for C-silylation is achieved by employment of the platinum-divinyltetramethyldisiloxane complex as catalyst [26]. The PEO-OH modified PDMS disks were removed and washed thoroughly with acetone, dried by N_2 and subsequently placed under vacuum for 12 h at room temperature.

2.5 Addition of *N*-succinimidyl carbonate (NSC) to PEO-OH modified PDMS surfaces

PDMS-PEO-OH was modified to incorporate an NHS ester for subsequent modification with the dendrimer or cell adhesion peptides as previously described (manuscript in preparation, see chapter 6). PDMS disks with surface grafted PEO-OH were placed in a solution of acetonitrile (1mL), triethylamine (0.1mL), and *N,N'*-disuccinimidyl carbonate (0.2g). The reaction vial was purged with nitrogen prior to being shaken for 6 h at 150 RPM. The disks were then washed with acetonitrile and diethylene glycol dimethyl ether and dried using N_2. The PEO-NSC modified PDMS disks were placed under vacuum for 12 h at room temperature and the surfaces examined by IR to evaluate functionalization.

2.6 Attachment of cell adhesion peptides to PEO modified surfaces

PEO-NSC modified PDMS disks were soaked in phosphate buffered saline (PBS, pH 5.5) containing either Arg-Gly-Asp-Ser (RGDS) or ^{125}I radiolabeled Gly-Tyr-Arg-Gly-Asp-Ser (GYRGDS) cell adhesion peptides, or Arg-Asp-Gly-Ser (RDGS) scrambled peptide at various solution concentrations for 2 h. Attachment of the peptide occurred via aminolysis by the amine terminus of the peptide sequence of the *N*-hydroxy succinimide ester located at the terminus of the surface grafted PEO chain. This reaction produces an amide linkage between the peptide and the surface bound PEO. The peptide modified PDMS-PEO disks were then rinsed thoroughly with buffer and dried under vacuum for 12 h at room temperature or counted for radioactivity.

2.7 Surface grafting of diaminobutane (DAB) dendrimer (G3).

PEO-NSC modified PDMS surfaces were placed in a vial containing diaminobutane dendrimer G3 in dichloromethane (CH_2Cl_2) and the mixture shaken for 6 h at 150 RPM at room temperature. The disks were then washed in CH_2Cl_2, dried using nitrogen and placed under vacuum for 12 h. The amount of dendrimer in the reaction solution was 16 fold molar excess relative to available terminal NSC ester groups per disk. Using the maximum surface peptide density achieved by the PEO-NSC linker system, as determined by radiolabeling with $Na^{125}I$, the amount of surface NSC groups was estimated by assuming a 1:1 (PEO-NSC:peptide) reaction resulting in the consumption of

all available NSC groups. Dendrimer was added in excess such that the theoretical number of PEO chains that may have attached to any single dendrimer molecule was minimized in order to achieve maximum availability of terminal amine groups for subsequent peptide grafting. A schematic representation of the reaction is shown in Scheme 1.

2.8 Attachment of cell adhesion peptides to dendrimer grafted surfaces

Attachment of cell adhesion peptides to dendrimer modified disks was achieved by first converting the peptide carboxylic acid groups to reactive NHS esters using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and NHS. A molar ratio of 5:5:1 (EDC:NHS:COOH of peptide) was used for reaction of EDC and NHS with the peptide in PBS buffer (pH of 5.5) for 24 h. Dendrimer modified PDMS disks were soaked in buffer containing either RGDS or ^{125}I radiolabeled GYRGDS cell adhesion peptides at selected reaction solution concentrations for 2 h. The PEO-dendrimer-peptide modified disks were then rinsed thoroughly with buffer and dried under vacuum for 12 h at room temperature or counted for radioactivity. Immobilization of the peptide to the dendrimer occurred via reaction of the peptide NHS ester with primary amino groups on the surface grafted dendrimer. Dendrimer modified surfaces were also soaked in peptide solutions sans EDC and NHS as a means for comparison with dendrimer surfaces containing covalently bound peptides. Peptide surface density was determined using GYRGDS for attachment and counting for radioactivity.

2.9 Radiolabeling of GYRGDS cell adhesion peptide

For quantification of peptide binding, the cell adhesion peptide with amino acid sequence Gly- GYRGDS was labeled using $Na^{125}I$ and IODO-GEN® iodination reagent (Pierce, Rockford, IL) as previously described. Separation of the peptide from unbound ^{125}I after iodination was accomplished using a reverse phase Sep-Pak C_{18} column (Waters, Mississauga, ON) containing a silica-based bonded phase of strong hydrophobicity, pre-equilibrated with 6-10 mL of methanol followed by 6-10 mL of 0.1% trifluoroacetic acid (TFA) in PBS buffer. The reaction solution was then poured into the column and rinsed with approximately 15 mL of 0.1% TFA in buffer. Solution fractions were measured for radioactivity using a portable handheld gamma counter (Surveyor M, BICRON, Solon, OH). Following elution of the unbound ^{125}I, the bound and labeled GYRGDS was eluted using a solution of acetonitrile and 0.1% TFA in PBS buffer (acetonitrile:0.1% TFA in PBS, 20:80 (v:v)). The peptide concentration of the most radioactive solution fractions was determined spectrophotometrically using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA). The most radioactive solution fraction of ^{125}I labeled GYRGDS was used for all subsequent dilutions.

2.10 Cell Culture

Human Umbilical Vein Endothelial Cells (HUVEC) (ATCC, Manassas, VA) were cultured in F-12K cell culture medium modified to contain 2mM L-glutamine and 1.5 g/L sodium bicarbonate. To this medium was added 0.1 mg/mL heparin (Sigma-Aldrich, Oakville, ON), 0.03 mg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich,

Oakville, ON), and 10% fetal bovine serum (Invitrogen, Burlington, ON). Cells were incubated at 37°C and 5% carbon dioxide in 75 cm^2 vented flasks (BD Bioscience, Franklin Lakes, NJ). Medium was replaced three times per week and cells were passaged at near confluence. Cells were passaged a maximum of four times prior to seeding on modified surfaces.

2.9 Cell Adhesion

HUVECs to be seeded on sample surfaces were grown to near confluence in cell culture flasks, subcultured and seeded on the surfaces in 24 well plates at a density of 25 000 cells/cm^2. The seeded surfaces were incubated for 2 h at 37°C to allow for cell adhesion followed by the addition of culture medium to each well. After 8 h, the culture medium was carefully aspirated and the seeded surfaces were rinsed gently by adding sterile PBS (3 x 250 μL). Cell number was determined fluorimetrically using the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR).

2.10 Data Analysis

Statistical analysis of data was performed using a one tailed Student's t-Test. A p value $\leq$ 0.05 was considered significant.

3 Results

3.1 Surface characterization

3.1.1 ATR-FTIR

The ATR-FTIR spectra of unmodified PDMS, as well as Si-H, PEO-OH, PEO-NSC, and PEO-dendrimer modified PDMS are shown in Figure 1. As expected, all spectra show the distinguishing peaks of the silicone elastomer substrate. However, features characteristic of each modification are clearly present. The spectra of surfaces modified with $(MeHSiO)_n$ contain a prominent peak at 2166 cm^{-1} representing the stretching vibrations attributable to the Si–H functional group. Following hydrosilylation, the Si-H peak has completely disappeared and there is an appearance of a broad CH_2 stretching vibration around 2850 cm^{-1} corresponding to the CH_2–O group on the PEO-OH, PEO-NSC, and PEO-dendrimer modified surfaces. A broad peak between 3700 and 3250 cm^{-1} is also visible and corresponds to the OH group present at the terminus of the PEO chain of PEO-OH modified surfaces. Following the addition of *N,N'*-disuccinimidyl carbonate to the PEO-OH modified surface, a sharp peak at 1742 cm^{-1} is visible and is attributable to the carbonyl of the ester linkage between PEO and the NSC group present at the terminus of the PEO chain. When the dendrimer is reacted with the PEO-NSC modified surfaces, a series of broad peaks appear between 1400 cm^{-1} and 1792 cm^{-1}. These peaks correspond to the carbonyl stretch of the amide bonds formed upon reaction of the terminal primary amines of the diaminobutane dendrimer with the reactive NSC ester groups on the surface grafted PEO. The broad CH_2 stretching vibration around 2850 cm^{-1}

corresponding to the CH_2–O group of PEO remains after attachment of the dendrimer and is overlapped by a broad peak between 2750 cm^{-1} and 2950 cm^{-1} corresponding to the C-H stretch of the CH_2 groups of the alkane chains of the dendrimer. A broad peak is also evident above 3000 cm^{-1} corresponding to the amine groups of the dendrimer.

These results clearly suggest that the PDMS disks have been successfully modified with $(MeHSiO)_n$, subsequently modified with PEO-OH via hydrosilylation and functionalized with NSC. Successful attachment of the dendrimer to the PEO-NSC modified surfaces is also confirmed.

3.1.2 Water contact angles

Advancing and receding sessile drop water contact angles were measured on PDMS, and PEO-OH, PEO-RGDS, PEO-dendrimer, and PEO-dendrimer-RGDS modified PDMS surfaces and are summarized in Figure 2. Unmodified PDMS showed characteristically high advancing and receding water contact angles (~107°) as expected. Modification of PDMS with PEO resulted in a reduction in the advancing water contact angle from 107° to 50°, and a reduction in the receding contact angle from 95° to 32°. These results clearly indicate a significant increase in surface hydrophilicity associated with surface grafting of PEO. Dendrimer modification resulted in an increase in the advancing and receding angles to 107° and 66°, respectively. Attachment of the RGDS peptide to the PEO modified surfaces resulted in an increase in advancing and receding water contact angles, from 50° to 73° and 32° to 57°, respectively.

Differences between the advancing and receding water contact angles for the control surface were generally small in comparison to the modified surfaces. This observation is likely a result of the smoothness and homogeneity of the silicone rubber in comparison to the relative inherent roughness and heterogeneous nature of the modified surfaces.

3.1.3 X-ray photoelectron spectroscopy

The elemental compositions of the surfaces, as measured by XPS, are summarized in Table 1. Following modification with PEO-OH, the C1s content increased and the Si2p content decreased at all takeoff angles indicating successful surface modification. Grafting of the RGDS peptide is confirmed by the appearance of elemental nitrogen at a 90° takeoff angle due to the presence of the amino groups of the peptide. Upon grafting of the diaminobutane dendrimer to the PEO-NSC modified surface, an elemental nitrogen composition of 5.6% is observed at a 90° takeoff angle, confirming the presence of the diaminobutane dendrimer.

High-resolution C1s spectra for the unmodified and modified PDMS surfaces at a 90° takeoff angle are summarized in Figure 3. The peak at a binding energy of 284.4 eV corresponds to the methyl carbons in PDMS. The small peaks at 286.3 and 288 eV are likely attributable to contamination. The peak at 286.5 eV visible in the PEO modified PDMS spectra corresponds to the ether carbon in the surface grafted PEO chain. The intensity of this peak is diminished on RGDS modified surfaces due to the reduced

presence of C-O bonds. Though the presence of C-N bonds from the peptide may also contribute to this peak, the net result is a reduction in peak intensity. Dendrimer modified surface spectra contain a very intense peak at 286.5 eV due to the high density of surface C-N bonds.

3.2 ^{125}I radiolabeled GYRGDS peptide surface grafting

Peptide surface grafting densities are summarized in Figure 4. Attachment of peptides to PEO-NSC and PEO-dendrimer modified surfaces resulted in trends of increasing surface grafted peptide with increasing peptide concentration in the reaction solution. Attachment of peptides to the PEO-NSC modified surfaces resulted in a maximum average peptide density of 60.2 pmol/cm^2 while the dendrimer modified surface resulted in a maximum average density of 77.7 pmol/cm^2. Therefore, a trend of increased surface peptide capacity via the dendrimer linker in comparison to the PEO linker at high peptide solution concentrations is apparent. However, dendrimer surfaces exposed to peptide solutions without EDC and NHS resulted in the greatest average surface peptide concentration of 124.9 pmol/cm^2. In this case, peptide absorption within the surface bound dendrimer is likely to have occurred. Increased steric interactions may suggest why peptide absorption is reduced on dendrimer surfaces containing covalently bound peptides via reaction with EDC and NHS.

3.3 Cell Adhesion

The cell adhesive nature of PEO-RGDS and PEO-dendrimer-RGDS modified surfaces were investigated by seeding HUVECs at a density of 25,000 cells/cm^2 and subsequently incubating for a period of 8 h. Percent cell adhesion of HUVECs on the sample surfaces is summarized in Figure 5. The lowest cell adhesion was observed on control surfaces containing no RGDS peptide, as expected. In general, PDMS control surfaces appear to have slightly better cell adhesive properties than PEO-OH modified surfaces, although the difference is not statistically significant. A trend of increasing cell adhesion with increasing surface concentration of RGDS peptide was observed on PEO-RGDS modified surfaces. The maximum average cell adhesion on these surfaces was 88.5%.

Cell adhesion on dendrimer-RGDS modified surfaces was not statistically higher than adhesion on the PEO-dendrimer control surfaces despite higher levels of peptide. Contrarily, cell adhesion on surfaces containing absorbed peptides alone (without the EDC linker) was significantly higher with a maximum average cell adhesion of 89.9% occurring at a peptide solution concentration of 15 μg/mL. Cell adhesion on PEO-RGDS surfaces was significantly higher than on PDMS-PEO and PDMS controls for all peptide reaction solution concentrations and was significantly higher than adhesion to surfaces containing the scrambled RDG modified surfaces at peptide solution concentrations higher than 10 μg/mL. This result clearly demonstrates that, cell adhesion peptides bound via the PEO spacer, are highly active and lead to a significant improvement in cell adhesion to the silicone elastomer.

Discussion

PDMS surfaces were modified with PEO by means of a platinum-catalyzed hydrosilylation reaction between the surface Si-H groups, generated by means of an acid-catalyzed reaction with $(MeHSiO)_n$, and allyl groups present on PEO. The successful creation of surface Si-H functional groups is evidenced by the high intensity Si–H absorption peak at 2166 cm^{-1} in the ATR-FTIR spectra of the PDMS elastomers modified by hydrosilane. The subsequent modification with allyl-PEO-OH was confirmed by the disappearance of the Si-H band in the ATR-FTIR spectra and the appearance of broad signals near 2870 cm^{-1}, corresponding to the CH_2–O of PEO, and between 3700 and 3250 cm^{-1} corresponding to the –OH functional group located at the terminus of the PEO chain. PEO-OH modified silicone elastomer surfaces were then functionalized with NSC by reaction with *N,N'*-disuccinimidyl carbonate. The success of this reaction is confirmed by the appearance of a sharp peak at 1742 cm^{-1} due to the presence of the NHS ester group. The absence of a broad peak between 3700 and 3250 cm^{-1} further indicates successful reaction.

Diaminobutane (G3) dendrimer was successfully grafted to the silicone elastomer surfaces via this PEO linker. This result is confirmed by the presence of a series of broad peaks between 1400 cm^{-1} and 1792 cm^{-1} in the ATR-FTIR spectra corresponding to the formation of amide linkages upon reaction of the primary amines of the dendrimer with the NHS ester terminal groups on the surface grafted PEO. Stretching vibrations around 2850 cm^{-1}, between 2750 cm^{-1} and 2950 cm^{-1}, and above 3000 cm^{-1} correspond to the

presence of the CH_2–O group of PEO, the CH_2 units of the dendrimer's alkane segments, and the dendrimer amine groups respectively.

Successful attachment of the PEO and diaminobutane dendrimer linker molecules was also confirmed using XPS. The presence of surface grafted PEO was confirmed by a decrease in the Si2p and an increase in the C1s content of the PEO modified PDMS. High resolution XPS showed a distinct peak with a binding energy of approximately 286 eV, corresponding to C–O, on all of the PEO-modified surfaces providing clear evidence that PEO was successfully grafted onto the elastomer surface. The appearance of an N1s peak on surfaces modified with RGDS via the PEO linker molecule indicates the presence of the surface bound peptide. PEO-dendrimer modified surfaces resulted in the appearance of an N1s peak corresponding to a surface nitrogen content of 5.6% resulting from the presence of nitrogen in the diaminobutane dendrimer structure.

Surfaces modified with PEO-OH also exhibited significantly increased wettability, as indicated by a decrease in the water contact angle compared to unmodified PDMS. Upon grafting of the diaminobutane dendrimer to the PEO modified surfaces, the water contact angle increased dramatically. This reduction in hydrophilicity indicates the successful attachment of the hydrophobic dendrimer to the previously hydrophilic PEO modified surface. A larger disparity between advancing and receding water contact angles is apparent on PEO-OH and PEO-dendrimer modified surfaces, likely due to increased surface roughness and heterogeneity.

Surface peptide densities were determined by radiolabeling GYRGDS peptide with ^{125}I prior to reaction with the PEO-NSC and PEO-dendrimer modified surfaces. Attachment of RGDS peptide via the PEO linker resulted in a significant improvement in cell adhesion compared to controls. Contrarily, attachment of the peptide via the PEO-dendrimer linker using EDC and NHS as a binding mechanism resulted in no significant increase in cell adhesion compared to controls. However, peptide density on the dendrimer modified surfaces (with EDC) was slightly greater than that achieved on surfaces containing peptides bound via the PEO linker at high peptide solution concentrations. Therefore, adhesion to the modified surfaces may not be a function of surface peptide concentration alone, but rather a combination of several factors which determine surface-bound peptide bioactivity. These determinants may include peptide spacing, mobility, orientation, and ligand clustering capability as defined by the polymer linker molecule. In this case, the dendrimer structure is comprised of a rigid structure of defined terminal group spacing with limited mobility for grafted ligands. It should also be noted that grafting of the peptide by means of a reaction with EDC and NHS results in attachment to the dendrimer linker via the peptide's C-terminus. However, the increased adhesion on peptide modified surfaces containing the PEO linker is presumably due to the high rotational capability of the PEO ether bond which promotes greater peptide mobility leading to more effective cell receptor interactions.

Cell adhesion on surfaces containing absorbed peptides alone (without the EDC linker) was significantly higher than on surfaces containing the covalently bound peptide. This result is somewhat unexpected, as soluble adhesion peptides have been previously shown to inhibit cell adhesion [27]. These surfaces demonstrated the greatest cell adhesion at intermediate peptide concentrations suggesting that an optimal absorbed peptide concentration for cell adhesion exists. This may be attributed to increased diffusion of unbound peptides into solution at high concentrations of absorbed peptide, potentially saturating the cell surface RGD receptors and hindering adhesion to the surface. However, the exact mechanism by which this increased cell adhesion is mediated is currently unknown and the subject of future investigation.

Dendrimer modified surfaces demonstrated the highest capacity for cell adhesion peptides. Interestingly, peptide density was the greatest on dendrimer surfaces containing absorbed peptides. This is presumably attributable to increased steric interactions in the presence of surface bound peptides generated via the EDC and NHS coupling mechanism which may inhibit further reaction or absorption of peptides within the dendrimer structure. Nevertheless, both dendrimer surfaces containing bound and unbound peptides demonstrated an increased peptide surface capacity in comparison to the linear PEO linker. However, the theoretical 16 fold increase in surface functionalization resulting from the attachment of individual dendrimer molecules to single surface NSC functional groups was not observed. Instead, we presume that steric interactions between dendrimers may have favored attachment of multiple PEO spacer molecules to a single

dendrimer. Therefore, future efforts will be directed towards highly controllable surface grafting techniques including stepwise dendrimer growth from the biomaterial surface by means of iterative coupling and deprotection schemes.

Surface grafted dendrimer polymers may be well suited for generating hyper-functional surfaces for attachment and delivery of a variety of biomolecules. The unique highly functionalized structure of dendrimers also make them promising for surface grafting of multiple biomolecules to a single biomaterial surface. With an increased surface binding capacity, combinations of various bioactive agents may be grafted to a biomaterial such that it may better control and direct a variety of cellular processes. However the use of a mobile linker may be necessary to improve cell interactions in order to achieve suitable ligand bioactivity. The large capacity of dendrimers for surface adsorption of peptides and small proteins may also provide an effective method for delivery and release of therapeutics from the biomaterial surface.

Conclusions

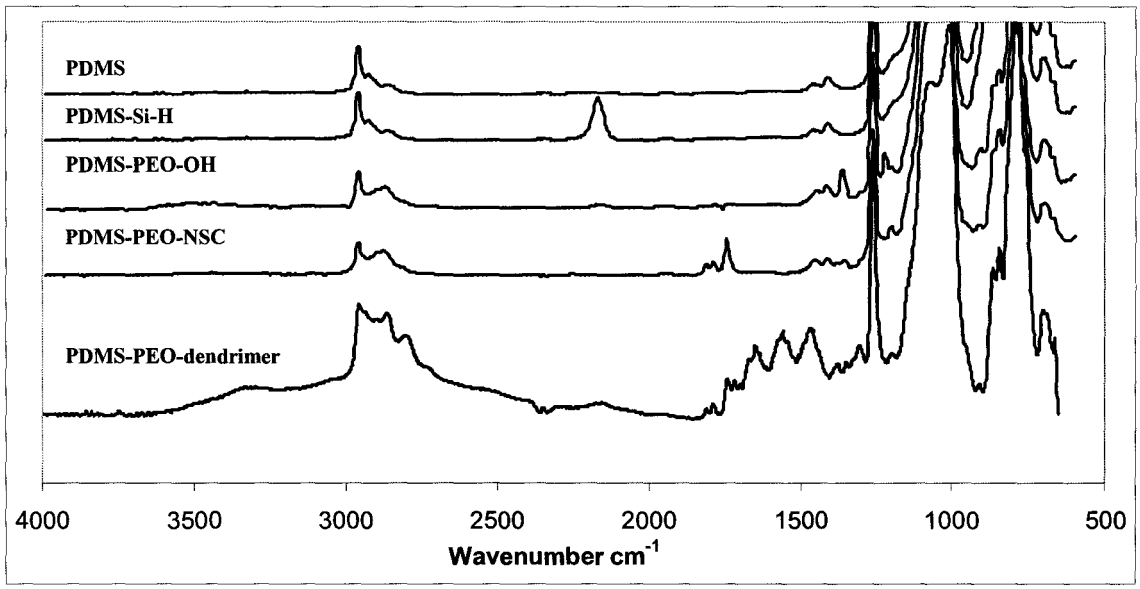
Surface grafting of dendrimers may be a promising technique for increasing the ligand binding capacity of biomaterials. In the present work, diaminobutane (G3) dendrimer was grafted to a silicone elastomer. An increase in surface grafted cell adhesion peptide was achieved on these surfaces compared to surfaces with peptides grafted via a linear PEO linker. However, concomitant increases in cell adhesion were not observed over the range of surface peptide densities on the dendrimer modified elastomers. Conversely, peptides grafted via the PEO linker alone gave a trend of increasing cell adhesion with increasing peptide surface density. Cell adhesion was also significantly higher on dendrimer surfaces containing adsorbed peptides when compared to the same surfaces containing covalently bound peptides. It is hypothesized that the spatial constraints of the peptides grafted to the dendrimer polymer may be responsible for the poor interaction with cell membrane integrins. Therefore, although cell surface peptide density may be an important determinant of cell adhesion, so too may be the spatial orientation and structural presentation of the surface bound peptide. Nevertheless, the dendrimer modified silicone elastomers provide a means for increasing the surface capacity of biomaterials for attachment of bioactive ligands. These modified surfaces may potentially be employed for examination of synergistic effects that may result from surfaces containing multiple ligands. The highly controllable synthesis of dendrimers may also provide the opportunity for precise control over surface grafted ligand spacing, mobility, distribution, and density.

Acknowledgements

Funding support from the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

List of Figures

Figure 1



Scheme 1

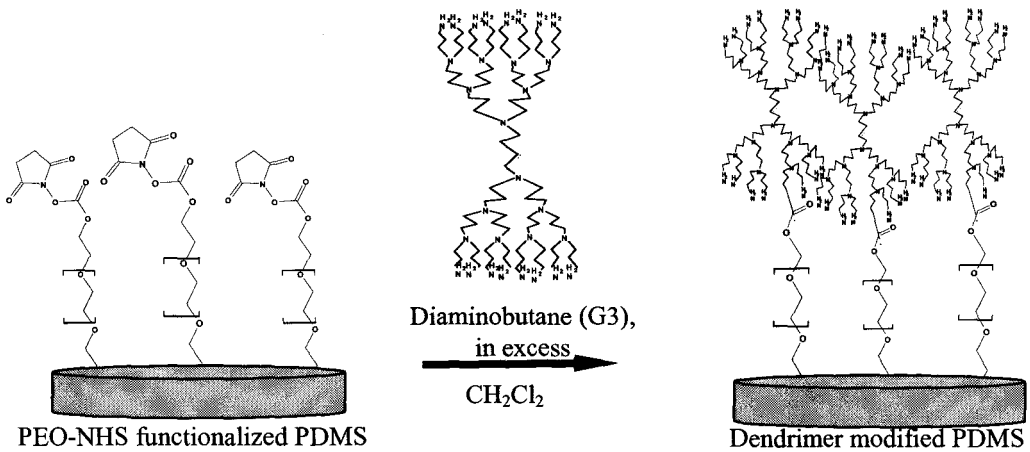


Figure 2

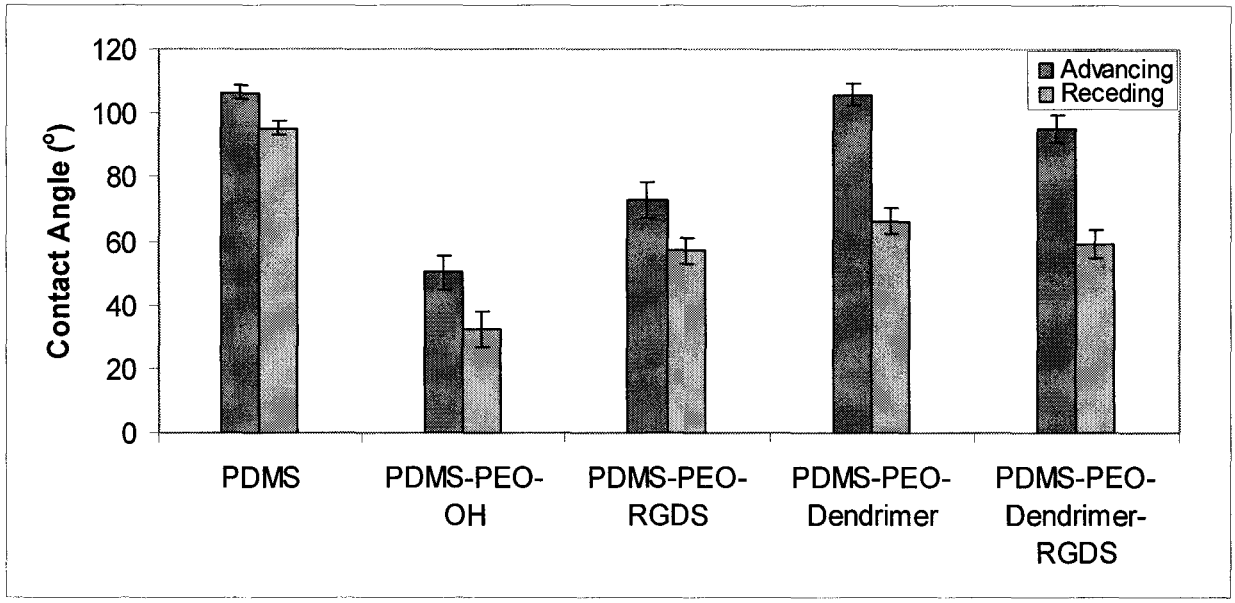


Table 1. XPS data showing % elemental composition of PDMS, PDMS-PEO-OH, PDMS-PEO-RGDS, PDMS-PEO-Dendrimer and PDMS-PEO-Dendrimer-RGDS surfaces.

Surface	Takeoff Angle (°)											
	90				30				20			
	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p	C1s	N1s	O1s	Si2p
PDMS	43.5	0	27.2	29.2	45.5	0	25.6	28.8	46.2	0	27.2	26.6
PDMS-PEO-OH	59.5	0	28.1	12.4	55.8	0	27.2	17.1	56.6	0	26.2	17.2
PDMS-PEO-RGDS	51.3	0.6	26.5	21.7	47.9	0	26.1	26	50.5	0	25	24.6
PDMS-PEO-dendrimer	58.2	5.6	25.2	11.1	53.8	2.4	26.1	17.7	52.4	1.5	25.8	20.3

Figure 3

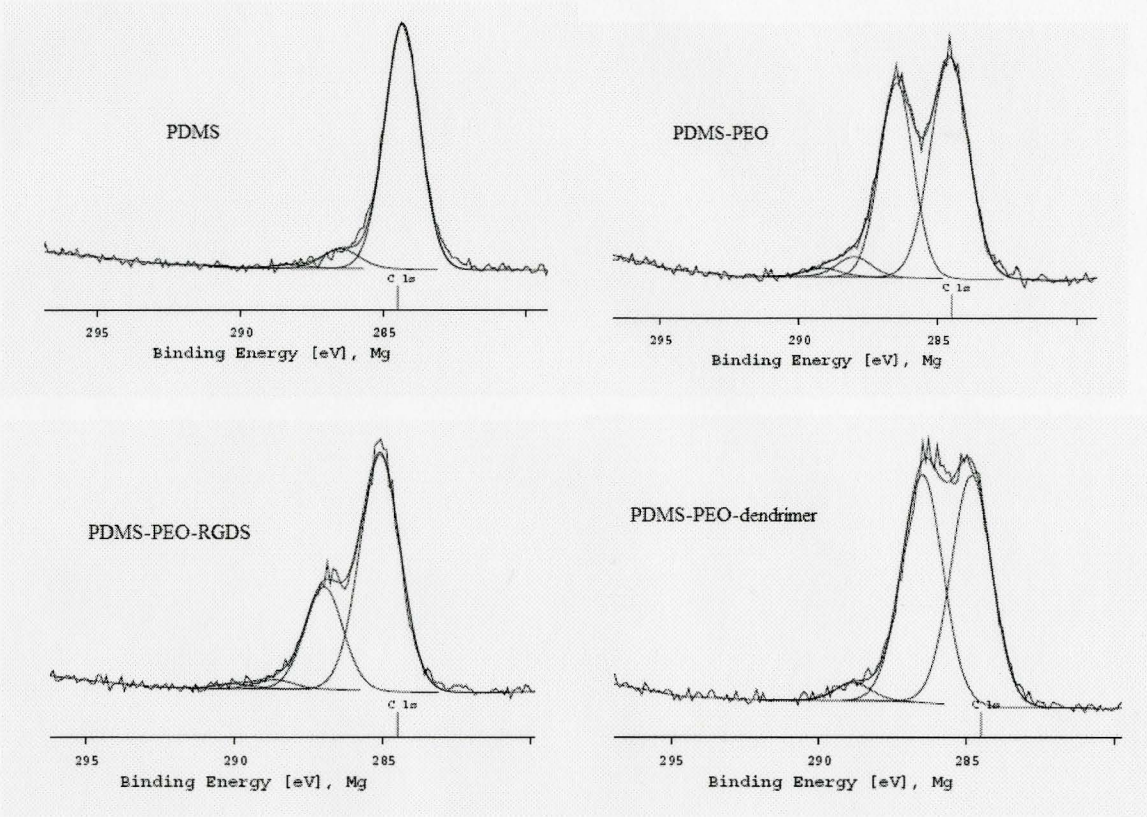

PDMS
C 1s
295
290
285
Binding Energy [eV], Mg
PDMS-PEO
C 1s
295
290
285
Binding Energy [eV], Mg
PDMS-PEO-RGDS
C 1s
295
290
285
Binding Energy [eV], Mg
PDMS-PEO-dendrimer
C 1s
295
290
285
Binding Energy [eV], Mg

Figure 4

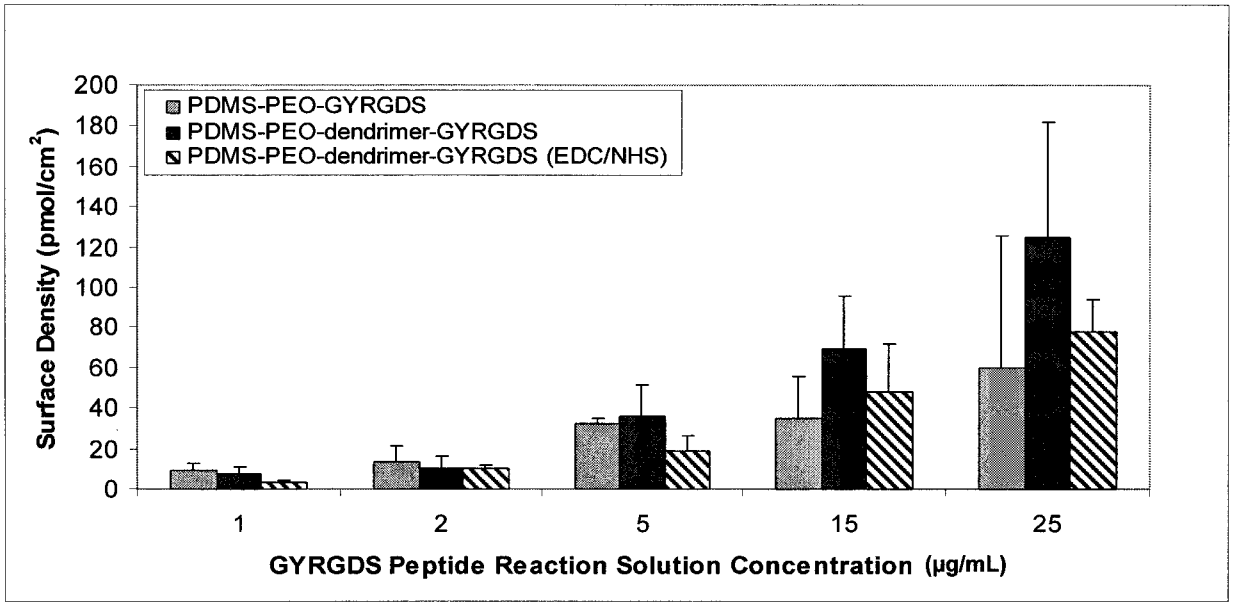
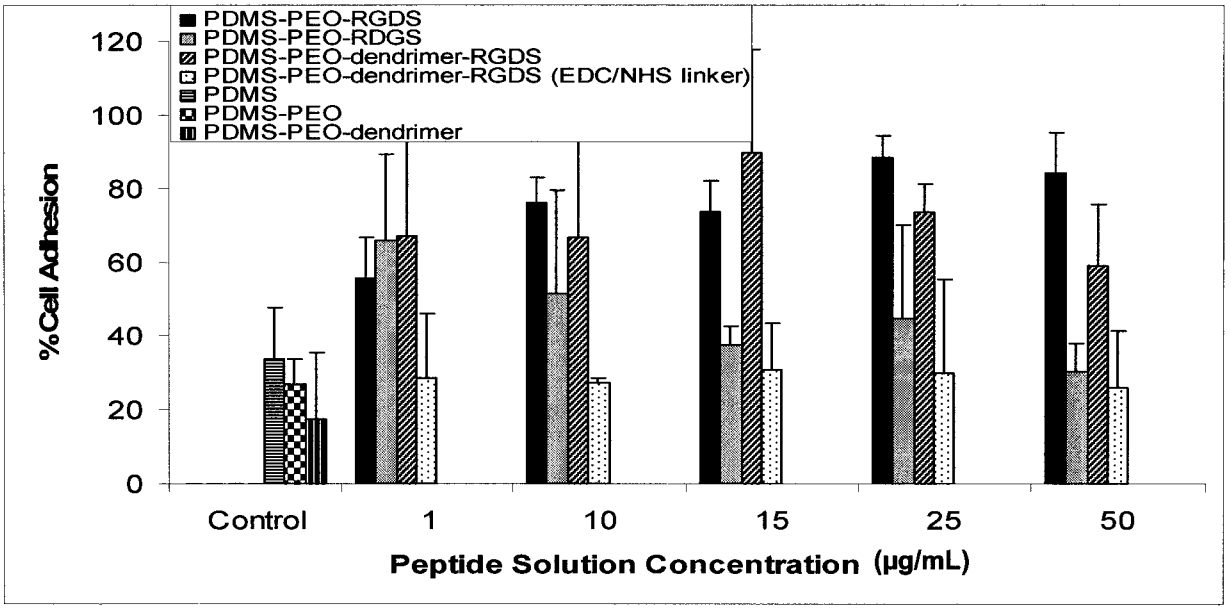


Figure 5



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9 CONCLUSIONS AND RECOMMENDATIONS

In this work, cell adhesion peptides were grafted to poly(dimethylsiloxane) (PDMS) via two polymer tether molecules. A novel robust method of modifying PDMS with functional PEO chains was optimized. Using this technique, PDMS was successfully surface modified with linear bifunctional allyl-, OH- terminated polyethylene oxide (PEO) chains (MW 550) and third generation diaminobutane (DAB) dendrimers. All polymer surface modifications were characterized by contact angle measurement, ATR-FTIR, and XPS. The PEO-OH modified surfaces were reacted to generate PEO-NSC surfaces. Cell adhesion peptides containing the RGD sequence were successfully grafted to PDMS via this PEO linker as well as via the dendrimer linker polymers. Quantification of surface peptide density, as determined using ^{125}I radiolabeled peptides, revealed an increase in peptide surface density on dendrimer modified surfaces when compared to surfaces modified with the PEO-NSC linker alone. This result suggests that the highly branched, hyper-functional nature of dendrimer polymers may be exploited for increasing the surface capacity of a biomaterial for attachment of biomolecules. However, cell adhesion to the dendrimer-peptide modified surfaces was reduced in comparison to the cell adhesion on PEO-peptide modified surfaces. Furthermore, a trend of increasing cell adhesion with increasing surface peptide density was observed on PEO-peptide modified surfaces; a similar observation was not made on dendrimer-peptide modified surfaces. Therefore, based on this work, it seems that the peptides bound to the surface via the highly mobile linear PEO linker showed improved

cell interactions compared to peptides linked via the rigid, highly branched dendrimer, despite the fact that higher peptide surface densities were observed on the latter surfaces. It is therefore hypothesized that ligand mobility on a biomaterial surface is a critical determining factor for effective ligand-cell receptor interactions. Therefore, cell adhesion appears to be a function of both surface peptide density and the structure of the peptide linker molecule. Dendrimer modified surfaces also exhibited a large capacity for absorption of cell adhesion peptides. This property may potentially be exploited for release of small proteins and pharmaceuticals from the biomaterial surface.

Protein adsorption to PDMS surfaces containing peptides grafted via the PEO linker molecule was also examined. ^{125}I radiolabeled fibrinogen adsorption to the modified surfaces was significantly reduced on both PEO and PEO-peptide modified surfaces when compared to PDMS controls. However, adsorption to the PEO-peptide modified surface was, not unexpectedly, greater than that observed on surfaces modified with the PEO linker alone. Nevertheless, this result demonstrates that the protein repulsive nature of PEO is partially maintained upon attachment of the bioactive peptide and that non-specific protein adsorption may be reduced by the use of this PEO linker chemistry. The PDMS surfaces modified with peptides grafted via the generic PEO linker molecule can therefore successfully decrease non-specific protein adsorption while increasing the surface capacity for cell adhesion. The straightforward method described in this work for high density attachment of biomolecules via a generic PEO linker molecule may be used for the generation of biologically relevant surfaces for a variety of applications.

A number of additional studies and future research objectives may be suggested in order to more fully examine the potential of these biomaterials for use in biomedical applications. In this work, dendrimer grafting was non-ideal, resulting in the attachment of multiple PEO spacer molecules to a single dendrimer. As a result, the theoretical increase in surface functionalization resulting from the attachment of a third generation dendrimer was not fully exploited. Further analysis directed towards determining the number of vacant amine groups remaining on the dendrimer after attachment of the peptide may give better insight into the grafting potential of these polymers. Moreover, more controlled surface grafting procedures should be explored for attachment of dendrimer molecules. This may include synthesis of the dendrimer prior to surface grafting, or stepwise growth of the dendrimer polymer on the biomaterial surface by means of an iterative coupling and deprotection scheme. Dendrimer modified surfaces may also be explored as a means for attachment of a variety of different biomolecules. These modified surfaces may potentially be employed for examination of synergistic effects that result from surfaces containing multiple ligands. Studies examining the effect of dendrimer generation on ligand bioactivity may also be of interest in order to gain precise control over ligand spacing and density. The dendrimer modified surfaces examined in this work also demonstrated a large capacity for absorption of peptides. Therefore these surfaces may be further examined and characterized with respect to their potential application for controlled release of drugs. The simultaneous use of dendrimer modified surfaces as vehicles for both attachment of biomolecules and localized delivery of drugs remains highly intriguing.

Future work may also be directed towards better elucidating the correlation between the structure of the polymer linker, ligand orientation, and resulting ligand bioactivity. This may be achieved by subtle modification of the linker molecule structure and ligand binding mechanism. For example, the effect of PEO linker chain length, and corresponding variations in ligand spatial mobility, on the bioactivity of surface grafted ligands may be explored. Dendrimer structure and generation may be similarly modified. Another potential research objective may include the exploration of dendrimer polymers as a means for increasing the surface capacity and coverage of PEO molecules. It is hypothesized that the generation of dendrimer-PEO-peptide surfaces will result in high density, highly bioactive surfaces. Furthermore, as all cell adhesion experiments in this work were conducted under static conditions, the effect of shear stress on cell adhesion should also be explored. An understanding of the effect of linker molecule structure on peptide bioactivity under fluid shear conditions is critical for the development of biomaterials for clinical applications. Finally, further biological testing may include quantification of various cell membrane markers of cells cultured on modified surfaces in order to gain better insight into the effect of biomaterial surface properties on cell behavior and phenotype.

10 APPENDIX A: SOLUTIONS AND REAGENTS

Phosphate-Buffered Saline (PBS)

Disodium hydrogen phosphate	1.32g
Sodium dihydrogen phosphate	0.345g
Sodium Chloride	8.5g

Fill to 1L with Milli-Q water. Adjust pH to 7.4.

Tris-Buffered Saline (TBS)

Tris	6.05g
Sodium Chloride	8.76g

Fill to 1L with Milli-Q water. Adjust pH to 7.4.

Glycine Buffer

Glycine	75g
Sodium Chloride	58.5g

Fill to 0.5L with Milli-Q water. Adjust pH to 8.8 with 2N NaOH.

Iodine Monochloride (ICl) Reagent

(a) Dissolve 150mg of $Na^{125}I$ in 8mL of 6N HCl.

(b) Dissolve 108 mg $Na^{125}IO_3$ H_2O in 2mL of Milli-Q water.

Mix (a) and (b) and bring the volume up to 40mL with Milli-Q water.

Add 5mL of CCl_4 and shake vigorously. Repeat until no pink colour remains in the organic phase.

Remove residual CCl_4 by aerating the solution for 1 h in a fumehood.

Bring the solution volume to 45 mL with Milli-Q water.

Prior to protein labeling, mix 1 part of stock ICl solution (as prepared above) with 9 parts of 2 M NaCl to achieve 0.0033 M ICl in 1.8 M NaCl.

11 APPENDIX B: DETERMINATION OF FREE IODIDE CONCENTRATION BY TRICHLOROACETIC ACID (TCA) PRECIPITATION OF PROTEIN

1. Dilute labeled protein solution, 1:10 (^{125}I labeled fibrinogen:PBS buffer).
2. Mix 100 μL of diluted labeled fibrinogen solution with 900 μL 1% bovine serum albumen (BSA) in Milli-Q water. Repeat in 3 separate vials, count radioactivity, and designate the average value as "A".
3. Repeat step 2. Add 500 μL of 20% TCA to 3 of the vials. Let stand for 10 min. Centrifuge at 3000 RPM for 1 min.
4. Count radioactivity of supernatant (equal volume as "A") and designate the average as "B".

Free Iodide (%) = (3*A)/B x 100