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**Synthesis of an Endothelial Cell Mimicking Surface
Containing Thrombomodulin and Endothelial Protein C Receptor**

By

Karl Erich Kador

A DISSERTATION

Presented to the Faculty of

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Under the Supervision of Professor Anuradha Subramanian

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Synthesis of an Endothelial Cell Mimicking Surface

Containing Thrombomodulin and Endothelial Protein C Receptor

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University of Nebraska, 2010

Advisor: Anuradha Subramanian

Synthetic materials for use in blood contacting applications have been studied for many years with limited success. One of the main areas of need for these materials is the design of synthetic vascular grafts for use in the hundreds of thousands of patients who have coronary artery bypass grafting, many without suitable veins for autologous grafts. The design of these grafts is constrained by two common modes of failure, the formation of intimal hyperplasia (IH) and thrombosis. IH formation has been previously linked to a mismatching of the mechanical properties of the graft and has been overcome by creating grafts using materials whose compliance mimics that of the native artery. Several techniques and surface modification have been designed to limit thrombosis on the surface of synthetic materials. One which has shown the greatest promise is the immobilization of Thrombomodulin (TM), a protein found on the endothelial cell membrane lining native blood vessels involved in the activation of the anticoagulant Protein C (PC). While TM immobilization has been shown to arrest thrombin formation and limit fibrous formations in *in-vitro* and *in-vivo* experiments, it has shown to be transport limiting under arterial flow. On the endothelial cell surface, TM is co-localized with Endothelial Protein C Receptor (EPCR), which increases PC transport onto the cell surface and increases PC activation via TM between 20-100 fold. This dissertation will

describe the chemical modification of medical grade polyurethane (PU), whose compliance has been shown to match that of native arteries. This modification will enable the immobilization of two proteins on an enzymatically relevant scale estimated at less than 10 nm. This dissertation will further describe the immobilization of the proteins TM and EPCR, and analyze the ability of a surface co-immobilized with these proteins to activate the anticoagulant PC. Finally, it will compare the ability of this co-immobilized surface to delay fibrous clot formation when compared to unmodified PU, albumin or heparin coated surfaces.

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**Introduction to Synthesis of an Endothelial Cell Mimicking Surface
Containing Thrombomodulin and Endothelial Protein C Receptor**

The design of biomimetic implants has led to the development of materials whose mechanical properties, 3D structure, adsorption or catalytic activity mimic those of the native tissue. The need for these types of materials is especially evident in the design of materials for vascular applications where mechanical property mismatch can lead to neointimal hyperplasia formation[1] and nonspecific protein adsorption[2, 3] and a failure to mimic the catalytic processes of the endothelial lining[4-6] can lead to thrombosis, occlusion and failure.

Chronoflex AR™, a medical grade polyurethane (PU), has been molded into grafts which have been previously shown to mimic efficiently the compliance of the native vessel seen in arterial flow.[7] However the use of these grafts has been limited by activation of the proteolytic clotting cascade that PU as well as other synthetic polymers exhibit which ultimately leads to the formation of emboli, occlusions and failure.[2] Several surface modification techniques have been designed to passivate the material surface through the immobilization of proteins or small biomolecules or polymers designed to reduce the hydrophobicity of the surface.[5] Others have attempted to create surface modifications which interact with the natural regulatory mechanisms of the body to prevent thrombin formation and occlusion. These modifications include direct thrombin inhibitors designed to mimic the effect of the enzyme hirudin found in snake venom[8] and immobilization of heparin[4, 9] or thrombomodulin (TM)[4, 10] found on the endothelial cell surface.

Thrombomodulin (TM) is a transmembrane glycoprotein found on all vascular endothelial cells.[11] In vivo, TM binds thrombin in a 1:1 ratio and acts as a cofactor for the activation of the anticoagulant Protein C (PC).[12] Once activated, PC reacts through competitive inhibition with Factor Va and Factor VIIIa. The inhibition of these molecules inactivates the Factor VIIIa / Factor IXa complex used to produce Factor Xa and the Factor Xa / Factor Va complex used to activate thrombin, the central molecule in thrombus formation.

TM has been coated or immobilized onto a variety of biomaterial surfaces including PEG-modified glass, polyethylene (PE), poly-tetrafluoroethylene (PTFE) and polyether-urethane (PEUU) and the bioactivity of immobilized TM was verified by biochemical assays. [10, 13-15] More recently, arterial grafts produced from transplanted veins have been studied and it has been shown that when the concentration of TM is increased from venous levels to that found in arteries, thrombin formation is completely arrested.[16] In another study, TM immobilized polyester vascular grafts were able to completely prevent hyperplasia formation which is normally associated with fibrous clot formation in vivo.[17] These results suggest that a TM immobilized surface could have the potential for completely preventing the thrombosis on the material surface which currently prevents the construction of artificial small diameter vascular grafts. However, surface coatings of TM do have limits as it has been shown that under flow conditions PC activation by TM becomes transport limited.[18]

The native regulatory mechanisms found on the endothelial lining can be extremely complex and often require multiple signals found on the cell lining. One such mechanism is the TM activation of the PC. In this mechanism, PC is bound to endothelial protein C receptor (EPCR) and presented to TM for activation. In the absence of EPCR, this reaction has been shown to be up to 86% less efficient.[19, 20] However, because the two proteins interact on the same PC molecule, EPCR and TM must be co-localized in a small area, based upon the size of the proteins, at a distance <10 nm.[21]

The requirement that the two proteins be immobilized on the 10 nanometer scale requires a degree of patterning which has been produced through different methods of lithography, however these methods have been developed for use on silica chips, which has not been yet been adapted for use on organic polymers.[22, 23] Because the ceramics do not have the necessary modulus and compliance for use in vascular systems, and the lithography methods which can be resolved to the 10 nm scale have not been adapted to use on polymer surfaces[24-26], a new method for ordered immobilization must be developed.

In chapter 1 of this dissertation, a chemical modification will be described to create a bi-dentate functional group capable of immobilizing two proteins on the necessary sub 10 nm scale. This chapter will discuss the synthesis and characterization of the modification done across the solid-liquid interface of a prefabricated sample of Chronoflex AR™ PU. In this chapter, the cellular and blood biocompatibility of modified polymer samples will

be compared to unmodified controls to ensure that these properties remain unchanged. Because of the importance of mechanical properties in the use of PU for vascular grafts, chapter 2 examines the mechanical properties of modified PU and compares modified and control samples in energy loss fatigue experiments. Chapter 3 gives a description of thrombosis and device initiated thrombosis. The chapter describes the TM / EPCR / PC anticoagulant mechanism and explains the production and purification of recombinant EPCR. This chapter also examines random immobilizations of TM and EPCR on a membrane surface analyzes. In chapter 4, we finally examine the effects of sequential co-immobilization of TM and EPCR on the ability of TM to activate PC. Also in chapter 4 we examine whether TM / EPCR immobilized surfaces are able to delay fibrous clot formation as examined by thromboelastograph. The final chapter discusses future directions for this research including the TM / EPCR surface and other possibilities for bi-dentate functional group.

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Chapter 1

Controlled Co-immobilization of Proteins on Polyurethane.

Abstract

Surface modification of implantable materials have been studied as a manner to passivate the material and create a more biocompatible implant. However in recent years, the systems used for surface modification have moved to systems which mimic that found on the cell surface. As a result of these more complex systems, methods have been developed to pattern the immobilization of biomolecules to create surfaces capable of the complex events which take place on the surface. These surfaces are limited by the need of a metal or silicon chip, which do not closely match the mechanical properties of soft tissue of methods which are not tuneable to the enzymatic scale for methods using polymers. This paper describes a method for sequential patterning of two proteins or biomolecules onto a polyurethane support whose mechanical properties closely resemble those of vascular tissue. This method creates a bidentate functional group capable of controlled immobilization of two different molecules, each of whose concentration is similar to those currently reported by immobilization on polyurethane through other methods.

Introduction

Implanted medical devices are of increasing importance in the practice of medicine and it is estimated that more than 3-million people in the United States have long-term implants like vascular grafts, pacemakers, catheters and joint replacements. Of particular interest is the design of small diameter vascular grafts as patients in need of arterial grafts for bypass operations do not have suitable autologous vessels.[27] Several types of materials have been tested in experimental configurations including initial studies where tubing composed metal or glass were used.[28] Today, the importance of matching the mechanical properties of the graft to those of the artery and its relationship to long term survival is known. Because of the elasticity and compliance of native arteries, development of artificial grafts has moved to polymers and recovered biological materials.[29]

Several different polymers have been used to generate vascular grafts. Dacron, a polyester (PE) and polytetrafluoroethylene (PTFE) have both been used in the design of large diameter vascular grafts. PE and PTFE grafts are porous and when exposed to continuous blood flow, surface induced thrombosis leads to the formation of clots around the pores, leading to the clogging of the pores and rendering a fibrin coated surface that allows the subsequent tissue ingrowth and partial endothelialization of the graft. The thrombogenicity which allows for the tissue ingrowth, and the mechanical properties, both parent polymers which have a significantly different stiffness and compliance as compared to a natural vessel, can lead to occlusion and intimal hyperplasia at the interface of the graft and the blood vessel in smaller diameter vascular grafts.[29] Efforts to design smaller diameter vascular graft through the use of bioresorbable polymers such

as polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone (PCL) and copolymers of these materials have been attempted. Initial studies showed difficulty in controlling the degradation rate, with constructs failing due to degradation prior to the formation of functional tissue. Copolymers have been employed in an effort to tailor both the mechanical properties and the degradation rate of polymer construct. The most successful biodegradable graft is polyglycolic-co-lactic acid (PGLA) / polydioxanone copolymerized graft which allowed for degradation over a period of 6 months and resulted in 100% patency over a 1 year experiment.[30] While this result shows promise, it is not yet viable for long term implantation. Another class of materials currently being studied for use in vascular grafts is biorecovered materials such as collagen, fibrin and decellularized extracellular matrix (dECM). Collagen composes a large concentration of native arteries, however the methods used to collect and process recovered collagen often leads to destruction of the tri-helix structure which gives collagen its natural strength and as a result collagen grafts have shown to possess poor mechanical strength. Fibrin scaffolds are not subject to the difficulties of recovery as they are polymerized in vitro and are of interest since fibrin clots are the natural scaffolds for vascular regeneration. To date fibrin constructs combined with tissue engineering techniques have shown better mechanical properties than collagen constructs, however they have not meet the mechanical strength of native vessels or been subjected to long term in vivo studies. dECM has been researched as a method of providing the mechanical properties necessary for implantation while removing the cellular components which would lead to immunorejection. While short term studies have demonstrated good cellular infiltration,

explanted devices have shown immunological reaction and intimal hyperplasia at the sites of implantation.

Polyurethane (PU) is an FDA approved biomaterial being studied for use in many cardiovascular implantable devices such as stents, vascular grafts, tissue engineered scaffolds[31, 32] and constructs and heart valves as its modulus and compliance closely approximate those of the natural blood vessel. The polymer also is of interest as it shows better biocompatibility, lesser immune response, better blood tolerance and better hydrolytic stability, than PE or PTFE and is more stable than the biodegradable constructs.[33] Briefly, PUs are actually copolymers polymerized through the reaction of a diol and an isocyanate. The resulting polymer is then classified into subclasses: as a poly ester urethane (PEsU), poly ether urethane (PEU), poly urethane urea (PUU) or poly carbonate urethane (PCU) based upon the bonds incorporated into its two subunits, however all urethanes contain the carbamate repeated subunit formed from the diol isocyanate reaction. Initial focus on biomedical devices was placed on PEsU because of the longtime use of polyester devices, however these materials were shown to have very short survival rates due to a high degree of biodegradation. PEU are the most commonly studied PU with several being available commercially for medical purposes. PCU are the newest class of PU being developed for medical implants, after concerns over PEU in long term implants (>10 years) were susceptible to oxidative degradation.

Two modes of failure have been closely associated with devices constructed from PU for cardiovascular applications, thrombogenesis and calcification. In an attempt to correct for these modes of failure, research has been conducted on the surface immobilization of different proteins and biomolecules. When these modifications are

more complex than physical absorption, they have required modification of the polymer either in the bulk phase, modification during fabrication or modification of the surface post fabrication. The most common method for modification of PU is the incorporation of a reactive group during the polymerization step. This method has been used to incorporate both amines[34, 35] and carboxylic acid[36-38] groups which could then be modified using standard coupling chemistries. While the number of the functional groups is controlled through concentration during polymerization, because the physical structure of the polymer is changed, the bulk properties of the material are also modified. It is also unclear in the case of amine incorporation if polymer becomes branched.[35] Modification of the carbamate amine (i.e. secondary nitrogen on the PU backbone) through the addition of a dibromo alkane extension has also been used to attach proteins and biomolecules.[39] The usage of the symmetric dibromo alkane along with the modification in the bulk phase, has the potential to cross-link the polymer chains and cause a stiffening of the bulk material. More recently, a bromo-thiol alkane has been used to prevent cross-linking and control the immobilization through the oxidation of a cysteine amino acid in the protein, though a draw back is that this linkage can easily be reduced in vivo.[40] Other methods have been developed to create reactive groups on the surface of PU post fabrication. The first method is a modification of the endcap of the polymer chain to produce an immobilization site. Because the synthesis only modifies sites terminal alcohols located on the surface of a device the bulk properties remain intact, but the method is limited by a low concentration of reactive sites exposed on the material surface. Treatment of fabricated polymer with plasma gases has been another effective method of functionalizing the surface. The plasma contains radical initiators

which break the surface bonds of the fabricated polymers surface and creates new functional groups on the surface which can be controlled somewhat by the gas used in the plasma and the gases used to quench the radicals on the surface. Several gases have been used in the plasma reaction including oxygen, ammonia, argon, methane, sulfur dioxide and different fluorocarbons. In the case of methane, the fluorocarbons and sulfur dioxide, the researchers attempted to use a one step process to create a more inert surface. Oxygen, ammonia and argon have been used most frequently to for surface groups for further immobilization. The functional group formed is somewhat controlled by the quench gas. Air quenching produces peroxides for oxygen initiation and hydroxyl groups for other initiators[41], ammonia quenching creates reactive amines[42] and acid quenching forms carboxylic acids on the surface.[43] While this method is advantageous because it can be use on a post fabricated surface, and reaction only penetrates a very thin surface with reactive surfaces ranging from 20 to 2000 angstroms,[44] there are downfalls to this method including a wide variety of functional groups are formed on the surface whose composition and concentration are not reproducible. Also to overcome low concentrations of functional groups, researchers have found it necessary to immobilize polyacrylic acid extensions.[41, 43] These extensions, which contain carboxylic acid groups bound to a central chain, are able to increase the number of functional sites, but this method does not ensure a uniform distribution of the immobilized molecule. The final method used to create reactive sites on polyurethanes is the hydrolysis of surface polymer chains. In this method, one to four normal sodium hydroxide is incubated with or without heat on a post fabricated sample allowing the base to cleave the chain bonds.[10] In this method the chains which are cut are held into the

sample through polymer chain entanglement and reactive sites, normally carboxylic acids can then be modified in a manner similar to the endcap chemistry. This method has the advantage of a high concentration of active sites formed, but severely weaken the sample should the reaction be allowed to occur over extend time.

To reduce the thrombogenicity of the PU, heparin, hirudin, albumin, thrombomodulin, different polysaccharides, chondritin sulfate and cholesterol[45] have been immobilized on the surface of implants. Other studies have attempted to endothelialize the surface of their constructs through immobilization of RGD containing peptides or different growth factors. In attempts to reduce the calcification, sulfonated polyethylene oxide[46, 47] and bisphosphonates[48] have been chemically immobilized onto the surface. Samples containing co-immobilization of different proteins and biomolecules have also been prepared in an effort to better mimic the endothelial surface, provide growth factors and binding sites for cell attachment[42] or in an effort to control multiple aspects of clotting[49]. However these co-immobilizations have only been controlled through concentration gradients during the immobilization steps[50] or the sequence in which the molecules are immobilized[42, 49].

Several techniques for patterning the immobilization of proteins or biomolecules in the design of biological sensors including photomask lithography, dip pen lithography / nanografting, “direct write” lithography and nano imprinting. In photomask lithography, a monolayer or polymer is coated either physically or chemically with photo-active polymer and a light source through a patterned cover immobilizes the target protein in the pattern on the surface.[51] This method is limited by low resolution on the mask and has been limited to micron and submicron patterns.[26] Recent work has

shown through molding methods that the distance can be reduced to 30 nm between wires, but more complex patterns or combining this with an immobilization step have not been undertaken.[25] Dip pen lithography, also known as nanografting, is a technique where the cantilever tip of an atomic force microscope is coated with the protein to be immobilized and it is touched to a reactive surface to create the covalent bond. The tip is then controlled to create the pattern on the surface.[22] This method of lithography offers some of the greatest control on the size and structure of the pattern, being able to create lines as thin as 3 nm and separations of lines on the same order.[23] Nanografting does have a considerable drawback in it is the method of patterning least able to be scaled up for industrial scale. In direct write lithography, an ion beam is used to burn through a chemically inert surface to expose a reactive surface beneath. In this case the immobilization target can be flowed over the surface for binding.[24] This method contains both of the drawbacks of the two previous types of lithography as it is based on the resolution of the laser and its ability for reorientation during the patterning process but is also not able to be scaled to commercially relevant scale. Nano imprinting is a method where a stamp is created through one of the other lithography and silicon etching processes but the proteins in question are not covalently bonded to the stamp but held until covalently bound to the stamped material.[52] This process allows for overcoming many of the difficulties in scale up as the mold can stamp many different samples[53], however the resolution of the proteins patterned on the surface and the alignment of the stamping have been shown to be difficulties which must be overcome.[52]

Jang et al. developed a very interesting method for patterning a surface sequentially which incorporated both a nanografting step with a chemical process. In this

method, a self assembled monolayer was created with two functionalities a reactive aldehyde and an inert diol.[54] A protein was attached to the reactive site through nanografting and followed by a reduction to convert the diol into a new reactive aldehyde. The new reactive sites could then be filled with the second protein. While this method showed the smallest resolution for coimmobilization, it requires a nanografting technique which can not be scaled and that the first protein immobilized will not be damaged by the redox reaction to activate the diols.

As a result of this review, we believe that there is a need to develop patterned surfaces so that more complex systems of antithrombogenic surface modifications can be studied for potential use in vascular systems. Because of the mechanical restrictions for therapeutic use, we believe that this system should be that these studies should be conducted on polymer implants rather than self assembled monolayers constructed on glass or metals which has been the source of most pattern immobilization research. From the review, it is important to develop a method that is repeatable and will allow for scaling to a manufacturing level rather than just single micrometer sized samples.

This research proposes a chemical method for ordered immobilization of two proteins or biomolecules onto a polyurethane sample. In this method, figure 2 and 3, a bidentate support will be synthesized from the surface of a post fabricated polyurethane sample across the solid / liquid interface. We hypothesize that the surface can be modified in a manner which allows for controlled immobilization to each functional arm of the bidentate support, one through a nucleophilic reaction and one through a coupling reaction, figure 4. We further hypothesize that by doing the reactions across the solid liquid interface, the bulk mechanical properties of the sample will be left intact. This

method will also allow for multiple samples to be modified in each batch rather than single sample batches which are required for most patterning methods.

Methods

Sheets of chronoflex AR medical grade polyurethane were provided as a generous gift from Cardiotech Inc. (Cambridge, MA).

2.1 Preparation of Surface Bridge

The chemical representation of the proposed surface modification scheme is shown in figure 2. Reaction 1 will enable the addition of the first reactive oxirane moiety to the secondary nitrogen incorporated in the carbamate subunit of the PU backbone. The addition of the reactive carboxylic acid branch of the bidentate bridge is achieved in reaction 2. Reaction 3 enables the addition of a reactive oxirane functional group to complete the bidentate bridge. Subsequent paragraphs will provide the experiment details of the surface modification.

2.1.1 Reaction 1

Solution cast polyurethane films were cut into 0.7 cm diameter samples with an average weight of 30 mg using a standard biopsy punch. Under an inert atmosphere, approximately 1 gram of polyurethane was added to 75 mL tetrahydrofuran (THF, HPLC Grade 99.9%, Sigma Aldrich, St. Louis, MO) mixed with 75 mL hexanes (ACS Grade, Sigma Aldrich, St. Louis, MO) and the temperature of the mixture reduced to -5 °C using an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized

with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.2 Reaction 2

PU samples from reaction 1 were added to a 0.02 M 5-amino valeric acid (Sigma Aldrich, St. Louis, MO) in water. The samples were allowed to react at room temperature for 24 hours with stirring. Samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times. All absorbed water was removed by evaporation under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.3 Reaction 3

Under an inert atmosphere, PU samples from reaction 2 were added to a 150 ml mixture THF and hexanes (50/50 by volume) and the temperature of the mixture reduced to -5 °C in an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours. Samples were stored at room temperature until further use.

Samples described in the above were prepared using a 50 / 50 by volume solvent mixture of THF and hexanes for reaction 1 and reaction 3. However, similar reactions were conducted using pure THF or pure hexanes as a solvent. Unless otherwise noted, all data described below has been collected from samples modified in the 50 / 50 (v) mixture of the two solvents.

Surfaces prepared through reaction 1, reaction 2 or reaction 3 were either subjected to surface analyses steps or subjected to protein coupling steps.

2.2 Chemical Analysis of Surface

2.2.1 Epoxide Quantification

Epoxide concentration was quantified as described previously.[55] Briefly, samples were prepared as described above through each reaction. Single samples were added to 12 mL 1.3 M sodium thiosulfate (pH 7) and allowed to react at least 6 hours with shaking, at which time a phenol red indicator was added. The samples were then titrated with 1 μ M hydrochloric acid to a visual endpoint. Samples were analyzed in duplicate from various batches.

2.2.2 FT-Infrared Spectroscopy

Samples were prepared from each reactive step and had all adsorbed water removed under reduced pressure. Spectra were prepared from multiple samples for each reaction as an average of 32 scans using an IlluminatIR FT-IR microscope (Smiths Detection, Danbury, CT). Sample spectra were normalized and subtracted out for comparison.

2.2.3 XPS Analysis

XPS analysis was provided by the National ESCA and Surface Analysis Center for Biomedical Problems located at the University of Washington. All XPS spectra were taken using a Kratos Axis-Ultra DLD spectrometer using a monochromatized Al Ka X-ray and a low energy electron flood gun for charge neutralization. X-ray spot size for these acquisitions was on the order of 300x700mm (Kratos 'Hybrid' mode). Pressure in the analytical chamber during spectral acquisition was less than 5×10^{-9} Torr. Pass energy for survey spectra (composition and detailed fluorine, nitrogen and calcium) was 80 eV and pass energy for high resolution C1s (HRC) scans was 20eV.

2.3 Mechanical Testing

Samples were cut to size (4 mm x 32 mm bars) and reacted as described above in THF, Hexane or a mixture of the two solvents (50% of each by volume). Samples were analyzed for stress at 37 C under a constant strain rate of 0.5 N/min to a strain of 5 N or failure using DMA Q800 (TA Instruments, New Castle, DE). The data was the analyzed as stress as a function of strain. The initial elastic modulus was found by calculating the slope of the data over the initial linear region.

2.4 Material Biocompatibility

2.4.1 Leachables

Samples prepared as described above, were added to Dubbelco's Modified Eagles Media (DMEM, Invitrogen, Carlsbad, CA) cell culture media at ratio of 1:3 w/v and incubated

for 3 days at 37°C with mixing. After incubation, samples were removed and discarded and the media, now termed as media with lechates (ML), stored at 4°C. Fibroblast cells (L929, American Type Culture Collection, Manassas, VA) were seeded on a 96 well polystyrene tissue culture plates and cultured overnight in 100 µL fresh DMEM media (FM) to allow for initial attachment. Following initial incubation, the cells were cultured in ML or control FM for 3 days before being released from the culture plate by the addition of 100 µL trypsin solution (0.05% trypsin in HBSS). The total number of cells was counted using a hemocytometer. Data is presented as a percentage normalized to the number of cells grown in FM.

Separate samples cultured as described above were analyzed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) viability test. Wells were rinsed with sterile phosphate buffer saline (PBS) to remove phenol red from the culture media. Fifty µl of MTT solution (1 mg/mL in phenol red free medium) was added to each well. Plates were incubated for 4 hs in CO₂ cell culture incubator, MTT solution was removed and 100 µl of isopropanol was added to each well. Plates were incubated for 10 min. in the incubator, followed by incubation for 15 min at room temperature. Absorbance at 595 nm was taken by a microplate reader (Elx800, Bio-Tek Instrument, Clarkston, MI, USA). Results were expressed in percentage of viability compared to control (% of control).

2.4.2 Hemolysis

200 µL citrated blood (Research Blood Components LLC, Brighton, MA) which had been diluted in a 4:5 (v/v) ratio with 0.9% saline was incubated with a 0.7 cm diameter

PU sample for 1 hour at 37°C with mixing. Following incubation, the samples were centrifuged at 700 x g for 10 minutes and the supernatant collected. The supernatant fluid was further diluted to 10 ml with the saline solution. The resulting solution was read by spectrophotometer at 545 nm. 100% hemolysis was set by incubation of the prepared blood with 500 µL of a 0.01 N HCl solution.

2.4.3 Thromboelastograph

Platelet poor plasma (PPP) was prepared from fresh, drawn less than 36 hours prior to usage and never frozen, whole citrated blood (Research Blood Components LLC, Brighton, MA) prior to each experiment by centrifuging whole citrated blood at a force of 2300 x g for 15 minutes at room temperature. Samples were inserted into disposable chambers which had been bored out to a diameter of 0.406 inches and 425 µL PPP was added. Clotting was initiated with the addition of a 200 mM CaCl₂ solution to a final calcium concentration of 6.0 mM. Samples were analyzed using a Thromboelastograph Analyzer 5000 (Haemoscope, Niles, IL) for speed of onset of the clot, initial formation rate of the clot and maximum strength of the formed clot.

2.5 Protein Immobilization

2.6.1 Protein 1 via Carboxylic acid

A 0.1 g / mL solution of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Thermo Fisher Scientific, Rockford, IL) was prepared in a 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES, Sigma Aldrich, St. Louis, MO) solution pH 4.5. 500 µL of the EDC in MES solution was added to each sample and was allowed to react

at room temperature with shaking. After 15 minutes, 500 μL of a 20 mg / mL *N*-hydroxysulfosuccinimide (Sulfo-NHS, Thermo Fisher Scientific, Rockford, IL) in MES buffer and added to each sample's solution. After 30 minutes at room temperature, the solution was removed from each sample and 1 mL of a 1 mg / mL solution of protein 1 prepared in phosphate buffered saline (PBS, pH7.2) was added to each PU samples from reaction 3 for 2 hours at room temperature. Upon completion, the PU samples were washed with 2 mL of PBS per sample 2 x 30 minutes.

2.5.2 Protein 2 via Oxirane

Following the immobilization of protein 1, 1 mL of a 1 mg / mL solution of protein 2 prepared in PBS was reacted for 24 hours at 4°C. The samples were washed 2 x 30 min PBS, 2 x 30 minutes PBS +.05% Tween 20, 3 x 30 min PBS.

Following final protein attachment the samples were stored in PBS at 4°C.

2.6 Protein Analysis

2.6.1 Protein Quantification by Bicinchoninic Acid Method

Immobilized protein was quantified using the bicinchoninic acid (BCA) method. Briefly samples were incubated with BCA solution and a copper sulfate solution (QPBCA kit, Sigma Aldrich, St. Louis, MO). Incubation in the presence of protein reduces the copper which reacts with the BCA which was quantified by spectrophotometer. Samples were incubated with 1 mL of BCA solution which was diluted with 1 mL of PBS (pH 7.2) for 3 hours at 37°C or overnight at room temperature. Protein concentrations were calculated

based upon a standard curve, points in triplicate, which was incorporated during every experiment. Absorbance at 595 nm was taken by a microplate reader (Elx800, Bio-Tek Instrument, Clarkston, MI, USA).

2.6.2 Protein Quantification by ^{125}I Labeled Protein

IgG and BSA labeled with radioactive iodine (^{125}I , half life 60 days, MP Biomedicals, Solon, OH) were diluted in a 1:50 ratio to unlabelled protein and immobilized as described above. Samples were thoroughly washed as described in the protein attachment protocol and the radioactivity measured using a LKB Wallac 1282 Compugamma CS Universal Gamma Counter (Perkin Elmer, Waltham, MA).

2.6.3 Immuno-Fluorescence

Samples following protein immobilization, human IgG through the oxirane and BSA through the acid, were incubated with 1.5 mL of a 1:1000 dilution of anti-human IgG conjugated to FITC or a 1:100 dilution of anti-BSA conjugated to Texas Red fluorescent label overnight at 4°C. Samples were washed with 2 mL PBS + 0.05% Tween 20 for 30 minutes and repeated 3 times followed by washing with 2 mL PBS for 30 minutes and repeated 3 times to remove unbound antibodies. Surfaces were developed using a Leica DM IL Fluorescent microscope.

Results

3.1 Chemical Modification of PU

The overall scheme for the surface modification of PU surfaces and the further functionalization of PU-surfaces with the proteins of interest is schematically represented in figure 2. Briefly, the PU surfaces were first reacted with LDA, followed by reaction with epibromohydrin to yield oxirane terminated surfaces. The oxirane functionalized surfaces were further modified with aminovaleric acid to immobilize proteins via the pendant carboxylic groups. The reaction with LDA and epibromohydrin was repeated to introduce a reactive oxirane group for the immobilization of protein-2. PU or its variants (carboxyl-PU) has been modified, either on the surface or in bulk, to allow the attachment of biologically relevant molecules. In most cases, the surface modifications have introduced active sites that allow for the immobilization of one protein per reactive site. Our studies seek to mimic the catalytic reaction that takes place on biological surfaces, which often requires proteins to be either co-immobilized on a single reactive site or anchored in a way such that the reactive pockets are in close proximity to catalyze biological reactions (figure 3). The first and third steps of the developed synthesis are based upon the dibromo alkylation steps of Alferiev and Levy, however the synthesis was altered to be reacted across the solid-liquid interface. This modification of the synthesis is made to be more suitable for manufacturing by allowing for modification of the material surface post modification. This difference was also designed to prevent the modification of the bulk mechanical properties by limiting the modification only to the surface of the material and by preventing cross-linking of the polymer chains which can occur when using a symmetric extension.

3.2 Analysis of Modifications to PU

3.2.1 Quantification of Epoxide on Surface

In order to quantify the concentration of oxirane groups added to the PU backbone, which is also a direct measurement of available reactive moieties, PU surfaces obtained at the end of reaction 1 and reaction 3 were assayed for total oxirane or epoxide groups and the values obtained are reported in figure 5. Reaction-1 and reaction-3 introduced 6.71×10^{-3} mmol of oxirane groups/cm² and 5.10×10^{-3} mmol of oxirane groups/cm², respectively. At the end of reaction-2, a residual amount of 8.34×10^{-5} mmol of oxirane groups/cm² were noted of PU surfaces, indicating a majority of the groups (99%) introduced in reaction-1 with amino-valeric acid to yield a reactive –COOH group.

3.2.2 FT-Infrared Spectroscopy

The chemical modifications were characterized by using fourier transform infrared spectroscopy (FTIR). Initial assignment of peaks for the spectra of modified PU samples produced only peaks assigned to the bulk materials due to the low concentration of surface groups in comparison to those of the bulk material, figure 6. As a result the spectra were normalized to the greatest peak and compared for differences. In the spectra for reaction 1 samples, figure 7, a strong new peak can be observed at 1200 cm^{-1} which is characteristic of species containing oxirane. In the spectra for reaction 3, figure 8, a large broad peak can be found between 3100 and 3500 cm^{-1} attributed to the acid group arm of the bridge and a new peak can be found at the 1200 cm^{-1} attributed to oxirane arm of the bridge.

3.2.3 XPS Analysis

The synthesis was further confirmed through the elemental analysis and carbon bond analysis studied in XPS. XPS is able to give better resolution of surface groups as it only characterizes to a depth between 10-250 Å (1-25 nm).[44] The elemental analysis, figure 9 and 10, shows an increase in the relative oxygen concentration following reaction 1 and reaction 3 which corresponds to the addition of epibromohydrin and its oxirane species. In addition a significant increase in nitrogen concentration can be seen with immobilization of 5-amino valeric acid through the oxirane formed in step 1 of the synthesis. In examining the relative concentration of C1s, figure 11, it is important to note that this method is unable to distinguish between carbon oxygen species and carbon nitrogen species of similar bond type. As we proceed through the first reaction and a large increase can be seen in at 286.7 eV corresponding to the addition of the first oxirane extension from the surface. In reaction 2, there is a significant increase in the two acid bonding species corresponding to the addition of the amino acid. There is a small decrease in the carbon oxygen / carbon nitrogen species which could be attributed to just a relative decrease in ratio due to the second step, or could be a result of oxirane opening without amino acid binding. If a small number of oxirane are opening prior to reaction with the amino acid this could suggest that the percent yield calculation would be artificially high, figure . This phenomena would not affect the final concentration of oxirane and total number of bi-dentate bridges formed, but instead would increase the percent completion of the final step of the synthesis. The XPS bond concentration analysis of the third step again shows an increase in the concentration of carbon oxygen

bonds while still maintaining the concentration of acid groups, suggesting that the bidentate bridge is formed and in the correct positions.

3.3 Mechanical Testing

Samples were analyzed for stress at a linear strain rate and plotted as stress as a function of strain. The initial slope was calculated as the initial modulus and charted as a function of the solvent used during the modification of the samples and shown in figure 12. Surface modification PU without impacting the bulk properties was a desired research goal, as a change in bulk properties may manifest as a decrease in the material properties of PU. Thus as an initial step, reactions were conducted in THF, pure anhydrous hexane and 50:50 mixture of THF and hexane to investigate the effect (if any) of the solvent used on the mechanical properties of the material. As noted in figure-X, , samples that had been reacted in THF had a significant change in the modulus in contrast to samples reacted in pure hexanes. This difference is attributed to the degree of absorption of the PU samples for the two solvent.

3.4 Biocompatibility

3.4.1 Leachables

The in vitro biocompatibility of surface modified PU films at the end of reaction-1, reaction-2 or reaction-3 prepared in the laboratory were evaluated to ascertain the impact, if any, of chemical crosslinkers or solvents used. While every step was taken to ensure the removal of reagents and solvents used in the modification steps, the impact of any potential leachables from the modified PU films was evaluated. The ability of

extracts or leachables, obtained from the incubation of modified PU surfaces in cell culture medium, to support growth and proliferation of fibroblasts is presented in figure 13 and 14. Similar cell counts were noted between fibroblasts cultured fresh medium and leachates. The absorbance from the cell viability test (MTT assay) on various PU films was calculated as a percentage of the control (cells on tissue-culture polystyrene, TCP). The viability of cells on the PU films was not significantly different from the cells on the TCP. The results show that the modified PU surface was not cytotoxic to the fibroblast cells, because the cell viability remained the same as the control after 3 days in culture. Cell viability in the range of 90–97% was routinely attained, indicating that modification is not cytotoxic and the modified PU samples maintained biocompatibility.

3.4.2 Hemolysis

Hemolysis testing is the most common method to determine the hemocompatibility properties of biomaterials and was evaluated because erythrocytes are among the first cells that come into contact with foreign materials in the blood vessels. Damage of red blood cells (hemolysis) by contact with biomaterial surfaces was used as measure of the biomaterial incompatibility and was determined by spectrophotometric measurement of hemoglobin content. The results did not demonstrate any hemolytic effects of modified PU surfaces, with the average hemolysis of both PU's under 2%. (figure 15)

3.4.3 Thromboelastograph

Thromboelastography (TEG) is a method of testing the efficiency of coagulation in the blood and measures the changes in elasticity at all stages of the developing and resolving clot. To evaluate the effect, if any, of the modified PU surfaces on the efficacy of coagulation, samples were studied by thromboelastography using platelet poor plasma (PPP). The time taken to clot onset, kinetics of clot formation, and strength of the clot formed were evaluated for modified PU films and compared to non-modified films and shown in figure 16. As noted in figure 17, no significant change was observed in the recorded times of initial fibrin formation (SP), time to clot initiation (R), or the strength of the clot formed using PPP, between control PU and modified PU samples.

3.5 Protein Immobilization

3.5.1 Quantification

While a variety of proteins can be immobilized on the modified PU surfaces developed in this study, to demonstrate the efficacy of protein attachment and offer proof-of-concept, the following proteins were chosen as model proteins: BSA and hIgG. The ability of each arm of the reactive bi-dentate functional group to covalently bind protein was quantitatively analyzed. The amount of covalently bound protein was quantified by two methods: a) a radiolabeled method in which ^{125}I -labeled protein were immobilized and the surface adsorbed radioactivity was gamma counted and b) by a colorimetric method that uses BCA to react with the available primary $-\text{NH}_2$ groups and reported in figure 18. The sensitivity of the radioactive method and BCA method is in the range of picomoles and micromoles of adsorbed protein, respectively. Values obtained by the independent methods are in good agreement. The total amount of covalently anchored

protein in $\mu\text{g}/\text{cm}^2$, which includes attachment on both the bi-dentate arms, was estimated to be in the range of $0.3 - 0.24 \mu\text{g} / \text{cm}^2$. Surface adsorbed protein density when protein was covalently attachment via one of the bi-dentate arms was estimated to be in the range of 0.1 to $0.16 \mu\text{g} / \text{cm}^2$. These concentrations are comparable to reported values of protein immobilization onto PU supports via incorporated carboxylic extensions or by plasma modification.

3.5.2 Immunofluorescence

To visualize surface adsorbed proteins on modified PU surfaces, either human IgG (hIgG) and bovine serum albumin (BSA) were first covalently attached through the desired mechanism and the surface bound protein was detected using a fluorescently tagged antibody. Representative fluorescent panels are depicted in figure 19. Panel-A, shows modified PU films that were sequentially exposed to FITC-labeled anti-human IgG and Texas-Red labeled anti-human BSA. The lack of fluorescent signal indicates the poor-reactivity of the secondary antibodies with the modified-PU films. Panel-B, shows modified PU films with covalently immobilized BSA via the $-\text{COOH}$ functionality (end of reaction-2) and exposed to FITC-labeled anti-human IgG and Texas-Red labeled anti-human BSA. The red color gives a qualitative measure of the presence of covalently immobilized BSA or an indirect measure of the success of the reaction-2 and its further reaction with BSA. Panel-C, shows modified PU films with covalently immobilized hIgG via the oxirane functionality (end of reaction-3) and exposed to FITC-labeled anti-human IgG and Texas-Red labeled anti-human HSA. The green color gives a qualitative measure of the presence of covalently immobilized hIgG or an indirect measure of the

success of the reaction-3 and its further reaction with hIgG. Panel-D, shows modified PU films with both BSA and hIgG covalently immobilized hIgG via the bi-dentate functionality and exposed to FITC-labeled anti-human IgG and Texas-Red labeled anti-human HSA. The presence of both the red and the green color gives a qualitative measure of the success of the reaction-2 and reaction 3 and its further reaction with BSA and hIgG, respectively.

Discussion

The combination of toughness, durability, biocompatibility, and biostability of polyurethane (PU) compositions has led to the usage of segmented PU in medical devices.[56] PU also contains essential features for a successful vascular prosthesis that has a low friction flow surface and optimal mechanical elastic properties similar to natural vessel.[57, 58] Thus biomedical grade PU was chosen a biomaterial surface. Previous methods have described methods of modification for the immobilization of proteins and biomolecules have included bulk and surface functionalizations that are limited to a single immobilized molecule. In more complex systems, multiple molecule immobilization may be required. The chemistry described above allows for the creation of bifunctional bridge extended from the surface which has been confirmed through FTIR and XPS spectroscopy. By utilizing this bifunctional bridge, two proteins can be attached to a single point on the materials surface in an enzymatically relevant scale. In this method, the attachment can be controlled as to which functional end of the bidentate bridge the protein is covalently bonded to, demonstrated in figure 19. Despite the addition of two proteins to the surface, each protein was immobilized at approximately the same concentration, figure 18, and a concentration similar to that of other PU immobilization methods, figure 1. Also of interest is that following modification, the PU samples were able to maintain the blood compatibility, figures 15-17, which makes the polymer of interest for vascular applications. By incorporating tetrahydrofuran (THF) as a solvent in the first and third step of the synthesis there was a significant lowering of the elastic modulus of the modified material as can be observed in figure 12. The choice of solvent was also changed after observing the effects solvent has on the mechanical

properties. The use of THF as a solvent for reactions using lithium diisopropyl amide (LDA) or sodium borohydride is common because the lone pairs of the bound oxygen help to stabilize the transfer of the metal during the reaction. However, samples which were reacted in pure THF were damaged during the wash steps in which THF has been absorbed into the polymer sample was removed using with deionized water. Ultimately these samples were deformed and failed under reduced loads, as can be observed in tensile testing data in figure. The THF solvent did serve two other important purposes in the synthesis, first it was able to remove any impurities that existed on the surface of the fabricated surfaces which can be observed by the near complete removal of all silicon impurities, which we speculate as being introduced by the casting of films on glass surfaces, by samples reacted with THF and only partial removal in samples reacted in hexanes alone. Secondly it allowed the samples to swell which created more absorbance of the reactants onto the surface to increase the yield of the reaction over samples reacted in hexanes alone. While the use of THF did reduce the modulus as compared to control PU, the samples modified in a 50/50 mixture were consistent with that of human aorta[59] and greater than that of veins used as arterial xenografts[60].

This new method of modification leads itself to several potential applications. Over the past two decades surface modifications have been made to the surface of polymer implants in an effort to passivate the surface, reducing the body's response to the material. However in recent years, more emphasis has been put on developing a biomimetic surface which is designed to interact with the body in a manner similar to natural tissue. The design of these surfaces is engineered to mimic the chemical composition of a cell membrane, the catalytic events on the cell surface, topography of

the natural surface, mechanical response of a tissue to a stimulus, or direct the adhesion and differentiation of cells. This has been especially evident in the development of suitable blood contacting surfaces for potential implants. Initially these surfaces were modified to change hydrophilicity or charge density before being modified with endothelial cell membrane groups such as heparin or thrombomodulin in first attempts to mimic the natural nonthrombogenic nature of endothelial cells. Due to the complexity of the coagulation pathways, current research is being conducted on the addition of multiple surface groups and blood proteins to inhibit the multiple aspects of clotting. In some studies, blood proteins such as albumin are used to passivate the recognition of the surface as foreign while coupling the surface coating with membrane molecule such as prostacyclin or heparin. In other attempts to create mimicking surfaces, a mixture of two membrane molecules such as heparin and thrombomodulin, are immobilized onto the surface of the material. Another technique used is to mimic the catalytic events which occur on the surface of endothelial cells such as the production of nitric oxide and inhibition of thrombin. However in all of these systems the coimmobilization of two different groups, the concentration and distribution on the surface are unable to be controlled. What is more of a concern is that in many of these cases the immobilization steps proceed sequentially using coupling chemistries which could couple one protein to the other damaging the first protein attached. In this case the damaged protein has the potential to unfold, losing the anti-thrombogenic qualities and instead becoming a point for the initiation of clotting. Coimmobilization via the bidentate bridge eliminates both of these concerns. In this method, the two proteins have equivalent distribution due to the tied immobilization site, and grafting the second protein to the first is eliminated by

conducting the fast coupling reaction first and a slow nucleophilic reaction second.

Because the nucleophilic reaction proceeds through a oxirane surface group not found on proteins or biomolecules, it is impossible to create the protein-protein grafting. Instead should a failure occur, it would be attachment of a single protein to multiple points of the surface.

The surface modification also provides the potential to activate enzymatic processes requiring two substrates. This process can be seen in the coagulation system in the activation of the anticoagulant Protein C (PC). In the activation of PC, a complex between thrombomodulin and thrombin is formed, which then clips the zymogen PC creating activated Protein C (aPC). In this example both thrombomodulin and thrombin could be immobilized through the bidentate functional group which could cooperatively activate PC. This process could also be designed in a system which more closely models *in vivo* aPC production by immobilizing thrombomodulin with endothelial protein C receptor (EPCR) a membrane protein which aids in the transport of PC to the membrane surface for activation. In this system, the kinetics of the catalytic surface are modified by changing transport characteristics of the PC, the rate limiting step of the anticoagulant's activation.

Endothelialized surfaces offer the potential of perfectly mimicking the compatibility of native blood vessels. Development of these materials has shown the need for two components, a point of attachment for the cell and a stimulus maintain the cells phenotype. While the attachment point can be as simple as a dimpled surface, the most successful have been the attachment of peptides which contain sequences derived from extracellular matrix proteins. The stimulus used to direct or maintain the phenotype

has varied between chemical and mechanical stimuli. For endothelial cells and their progenitors, the most common chemical stimulus is vascular endothelial growth factor (VEGF). Since VEGF acts through a receptor mediated process, its coimmobilization with a cell anchoring peptide can lead to proper attachment, spreading and phenotype. Another common stimulus is shear mechanical force of blood over the cell layer. While the laminar flow over the surface is needed for proper spreading, prior to the cells forming a complete monolayer, small clots can form on the surface in areas where cells did not properly attach. These thrombi can lead to the formations of eddies which disrupt the flow over the cells leading to improper remodeling of the surface. Should heparin or a similar anticoagulant be immobilized onto the surface with a cell binding peptide, the surface induced thrombi could be prevented leading to a more properly formed endothelialized layer. Thus in the present study, we were motivated to generate biocompatible surfaces that would enable the immobilization of two biomolecules or moieties per site of activation.

Conclusions:

With the growing development of biomimetic surfaces, methods of controlled coimmobilization of proteins onto surfaces have been studied. To date, these methods have been limited to self assembled monolayers, whose mechanical properties do not match those of the native tissue. As a result there is a need for a method to pattern immobilize proteins or biomolecules onto a polymer surface.

We have demonstrated a method to create a bidentate functional group extended from the surface of polyurethane which does not alter the biocompatibility of the surface. Using this method, we have demonstrated that by using the bidentate functional group we can control to which terminal of the bridge a protein is attached and that the concentration of each protein bound to the surface is similar to concentrations of proteins immobilized by other methods. Finally, we have shown that after modification the sample's mechanical properties are comparable to native tissue suggesting that the synthesis can be used for future design of biomimetic materials.

Reference	Protein / Biomolecule	Method of Addition	Concentration
Nojiri ASAIO Trans 1988	Heparin	Isocyanate extension	0.65 $\mu\text{g} / \text{cm}^2$
Lui J Biomed Mat Res 1993	(Insulin / transferrin) + collagen	Plasma Ammonia	Insulin 0.31 $\mu\text{g}/\text{cm}^2$ Trans 0.33 $\mu\text{g}/\text{cm}^2$ Coll 0.36 $\mu\text{g}/\text{cm}^2$
Kishda Biomaterials 1994	Thrombomodulin	hydrolysis	<1 $\mu\text{g}/\text{cm}^2$
Lee J Biomed Mat Res 1996	Collagen	carboxy PU	24 $\mu\text{g} / \text{cm}^2$
Phaneuf J Biomat App 1997	BSA / Hirudin	carboxy PU	300 ng / cm^2
Bae Biomaterials 1998	PEO / Heparin/ Acrylic Acid	Plasma O ₂	AA 0.47 $\mu\text{mol}/\text{cm}^2$ PEO 42 nmol/ cm^2 Hep 1.3 $\mu\text{g} / \text{cm}^2$
Phaneuf Artificial Organs 1998	BSA / Hirudin	carboxy PU	184 ng / cm^2
Huang J Biomed Mat Res 1998	Collagen	carboxy PU	25 $\mu\text{g} / \text{cm}^2$
McClung J Biomed Mat Res 2000	Lysine	Photo	3.2 nmol / cm^2
Van de Velde Biotech Bioeng 2000	Peroxidase + Glucose Oxidase	Entrapment during polymerization	NA
Aldenhoff J Biomed Mat Res 2001	Persantin	UV	20 nmol / cm^2
Alferiev J Poly Sci 2001	Bisphosphonate	dibromo extension carbamate nitrogen	0.09 mmol / g bulk mod
Alferiev Biomaterials 2002	dansyl cysteine	thiol extension carbamate nitrogen	0.45mmol / g bulk mod
Hsu Artificial Organs 2003	RGD containing Protein	gelatin entrapped polymer	NA
Yuan J Biomat App 2003	Sulfobetamine	Isocyanate extension	NA
LV J Mat Sci Mat in Med 2004	Acrylic Acid / Sulphonic Acid	Plasma O ₂	NA
Alferiev Biomacromol 2006	Heparin	dibromo extension carbamate nitrogen	1.11 $\mu\text{g} / \text{cm}^2$
Du J Biomed Mat Res 2007	AT3 / Heparin	AIBN off PUU	NA
Aksoy J Bioact Compat Poly 2008	Heparin	Plasma O ₂ / AA	NA
Sartori Reac and Funct Poly 2008	Acrylic Acid / Gelatin / polylysine	Plasma Argon	NA

Figure 1. Covalent immobilization of biomolecules onto a Polyurethane Support

Description of the method of modification, the molecule immobilized and the relative concentration of the immobilized molecule for biologically active polyurethane surfaces.

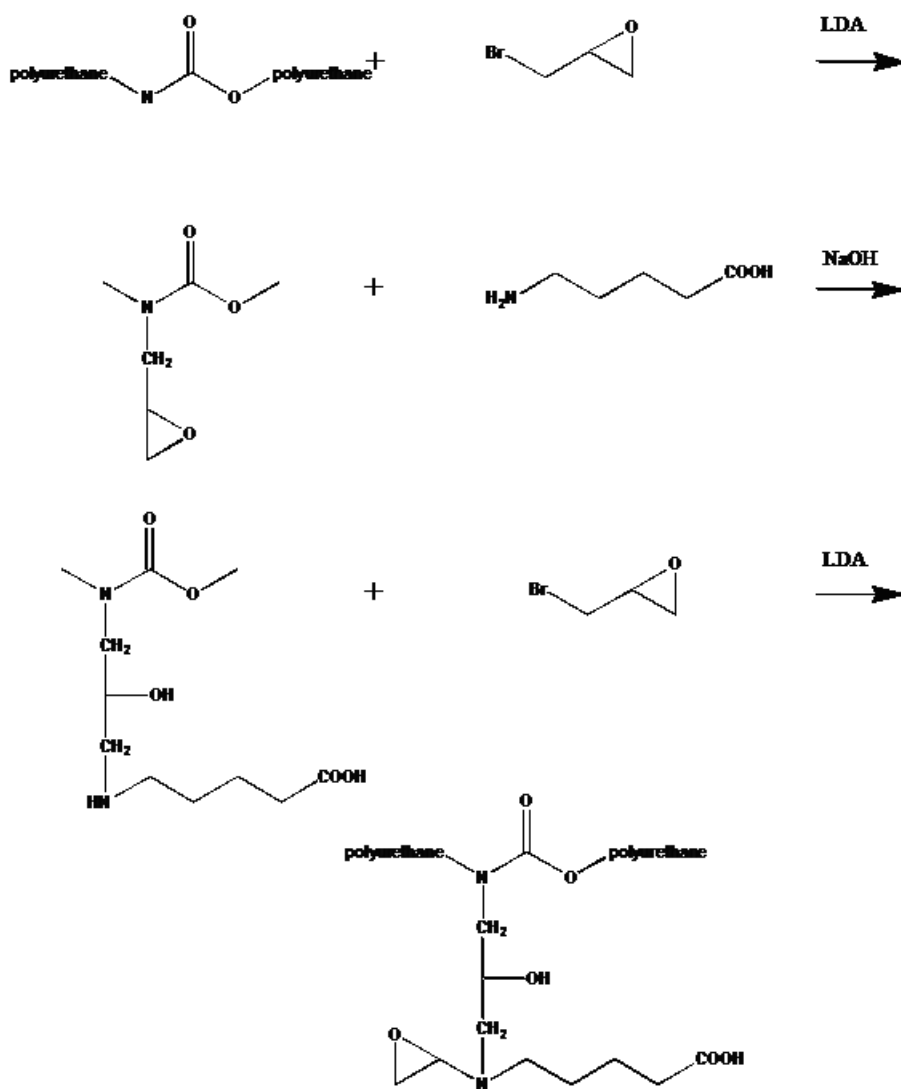


Figure 2. A schematic representation of the proposed three step chemical modification to produce bidentate bridge functional group on a solid polyurethane surface. Reaction 1 enables the addition of the first reactive oxirane moiety to the secondary nitrogen incorporated in the carbamate subunit of the PU backbone. The addition of the reactive carboxylic acid branch of the bidentate bridge is achieved in reaction 2. Reaction 3 enables the addition of a reactive oxirane functional group to complete the bidentate bridge.

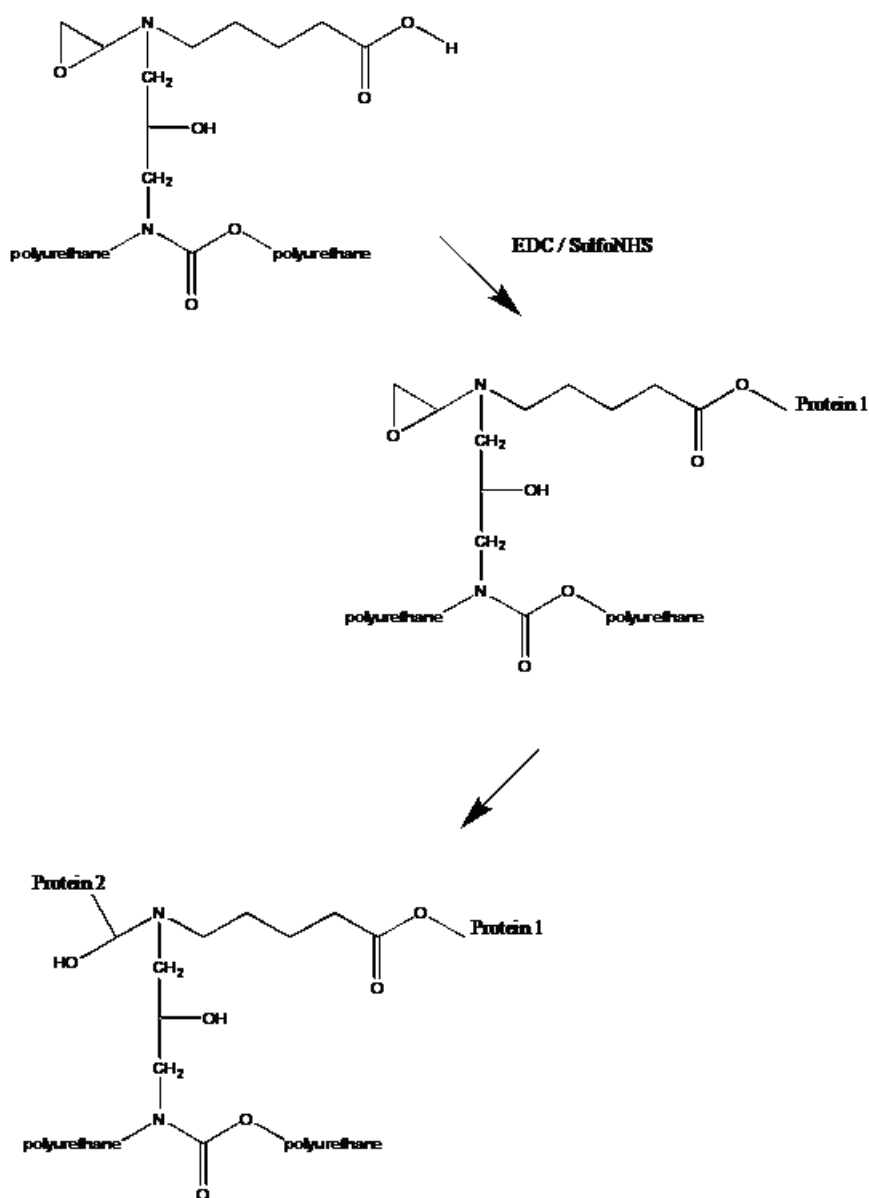


Figure 3. Sequential Immobilization of Proteins. Chemical representation of the sequential immobilization of two proteins onto polyurethane modified with the bidentate functional group. The pendant -COOH moiety will be first reacted with EDC/NHSS to generate a reactive ester and protein-1 will react via -NH_2 groups to form a stable amide bond. Protein-2 will be immobilized via the ring-opening of the oxirane moiety.

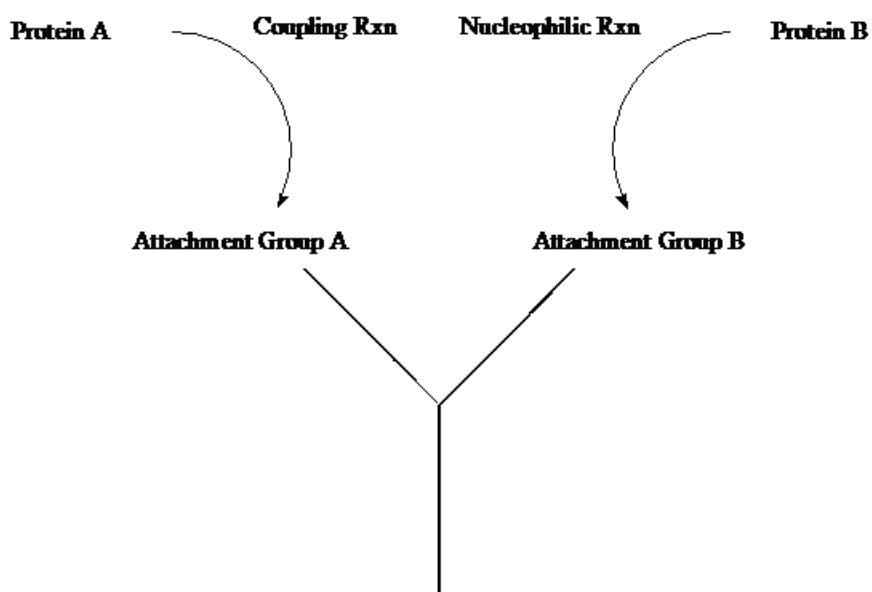


Figure 4. A schematic of the sequential immobilization strategy. Characterization of bidentate functional group and specifically the methods of attachment of each protein to the bridge. Protein-1 can be anchored via a nucleophilic reaction and protein-2 can be anchored via a coupling reaction.

	Average mmol of oxirane / cm ²	Standard Deviation	Percent Completion
Reaction 1	6.71E-03	1.39E-03	
Reaction 2	8.34E-05	1.15E-04	98.8
Reaction 3	5.10E-03	1.44E-03	76.1

Figure 5. Concentration of Oxirane groups on the Polyurethane Surface

Concentration of oxirane groups on the surface of polyurethane was calculated by titration following the ring opening reaction with 1.3 M sodium thiosulfate solution.

Percent completion is calculation based upon number of oxirane created (Reaction 3) or lost (Reaction 2) in comparison to the previous reaction. Samples were analyzed in duplicate from a minimum of three batches.

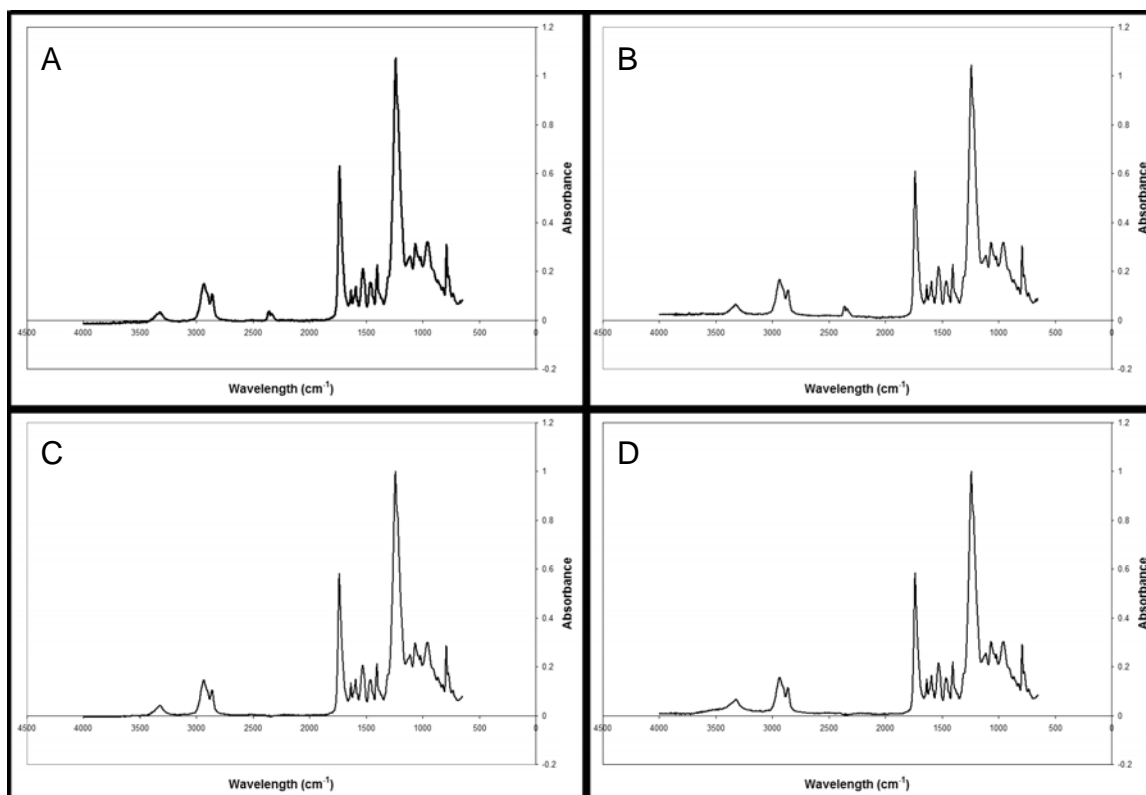


Figure 6. FT-IR Analysis of Polyurethane Samples.

FT-IR spectra of control samples of Chronoflex AR polyurethane (A) and PU modified through Reaction 1 (B), Reaction 2 (C), and Reaction 3 (D). No new peaks were able to be observed through the modifications due to the low concentration of functional groups formed on the surface in comparison to the chemical groups found in the bulk polymer. Peaks at 2300 cm^{-1} can be disregarded as carbon dioxide.

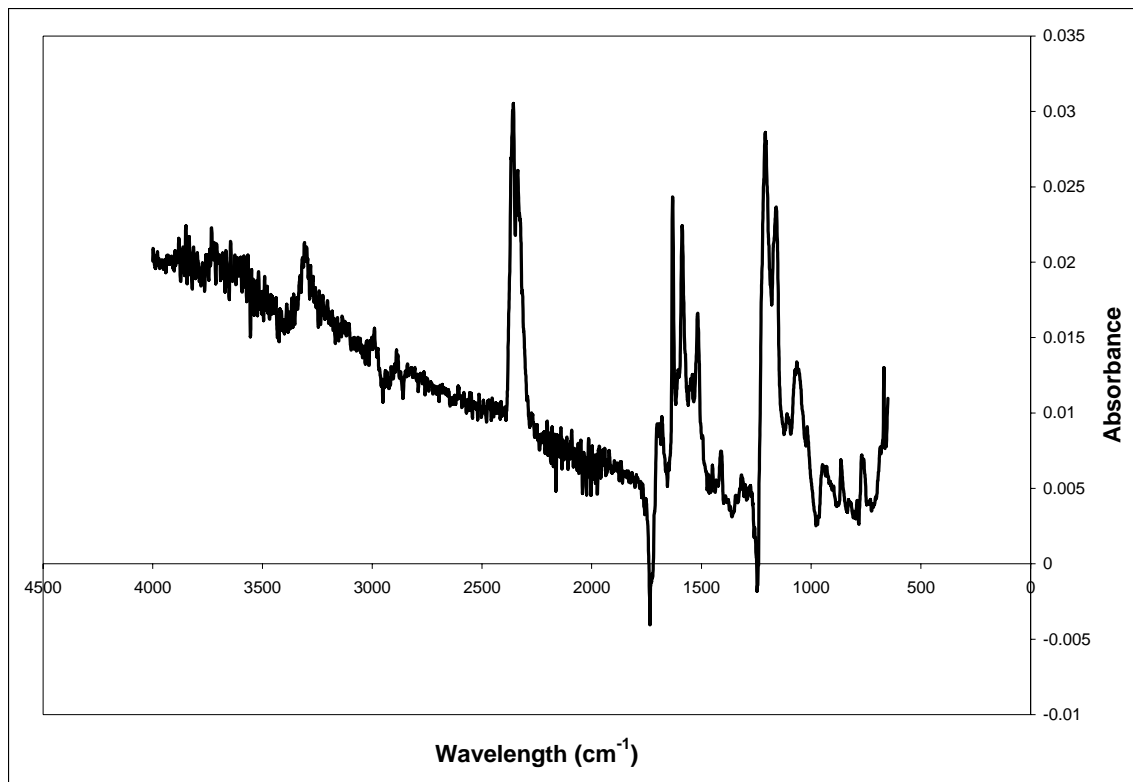


Figure 7. Differences of FT-IR Spectra of PU Following Reaction 1

Differences of FT-IR spectra of PU samples modified through Reaction 1 and spectra of unmodified Chronoflex AR PU. The formation of a strong new peak can be observed at 1200 cm^{-1} which is characteristic of species containing oxirane functional groups. Peaks at 2300 cm^{-1} can be disregarded as carbon dioxide.

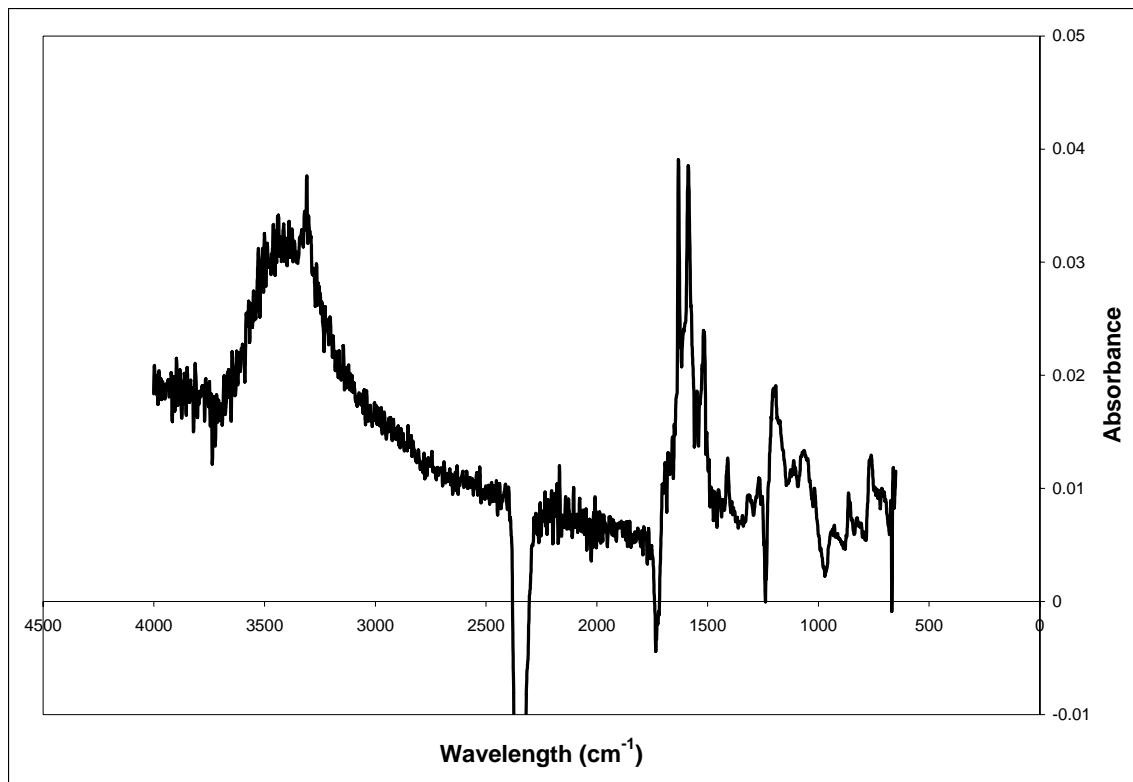


Figure 8. Differences of FT-IR Spectra of PU Following Reaction 3

Differences of FT-IR spectra of PU samples modified through Reaction 3, now containing the entire bidentate functional group and spectra of unmodified Chronoflex AR PU. The formation of a large broad peak can be found between 3100 and 3500 cm^{-1} attributed to the acid group arm of the bridge and a new peak can be found at the 1200 cm^{-1} attributed to oxirane arm of the bridge. The decrease in the relative strength of the oxirane peak is attributed to the percent yield of the modification. The peak at 2300 cm^{-1} can be disregarded as carbon dioxide.

Sample	C	O	N	Si	Ca	F
Control PU	68.5	18.9	0.7	11.4	0.5	N/D
Reaction 1 Hexane	71.7	21.2	1.7	4.7	0.2	0.6
Reaction 2 Hexane	69.2	21.5	2.9	5.0	N/D	1.5
Reaction 3 Hexane	66.9	23.6	3.0	4.5	N/D	1.9
Reaction 2 THF	75.0	22.3	1.7	1.1	N/D	N/D
Reaction 3 THF	75.5	22.1	1.6	0.6	N/D	N/D

Figure 9. Elemental Analysis of Polyurethane Surface by XPS

Elemental analysis of control Chronoflex AR PU films and films modified in hexanes or THF by XPS.

Sample	C	O	N
Control PU	77.8	21.5	0.8
Reaction 1 Hexane	75.8	22.4	1.8
Reaction 2 Hexane	73.9	23.0	3.1
Reaction 3 Hexane	71.6	25.2	3.2
Reaction 2 THF	75.8	22.5	1.7
Reaction 3 THF	76.1	22.3	1.6

Figure 10. Elemental Analysis of Polyurethane Surface by XPS without Silicon Contamination

Elemental analysis of control Chronoflex AR PU films and films modified in hexanes or THF by XPS. Elemental percentages were recalculated without silicon impurity.

Sample	Bond Energy eV			
	285.0	286.7	288.7	290.6
Control	89.9	6.9	2.2	1.1
Reaction 1	69.5	21.7	2.3	1.3
Reaction 2	68.7	18.8	7.1	5.4
Reaction 3	65.6	21.7	7.6	5.0
Associated Bonds	C-C/C-H	C-O	C-(C=O)-O	O-(C=O)-O

Figure 11. C1S Percentage by Bonding Energy

Percent of carbon bonds measured at each bond energy by XPS in control and modified polyurethanes.

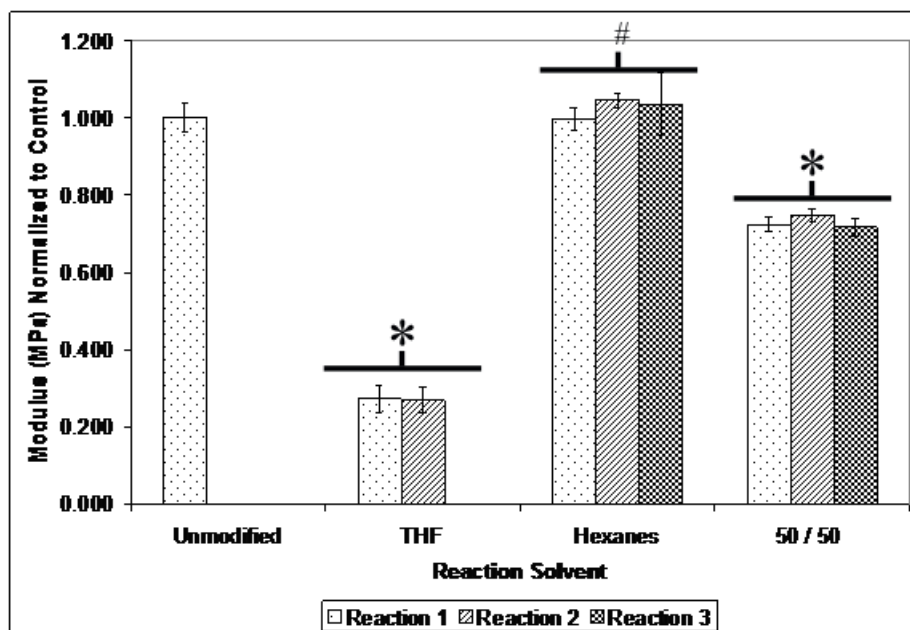


Figure 12. Mechanical Analysis of Polyurethane Samples.

Initial modulus of control and modified polyurethanes as a function of solvent system used during modification. Modulus was determined in tension with a constant strain rate of 0.5 N / min at 37°C. Sample modulus was normalized to the average modulus of control unmodified Chronoflex AR. Average modulus of control, 8.695 ± 0.319 MPa. Error bars represent one standard deviation.

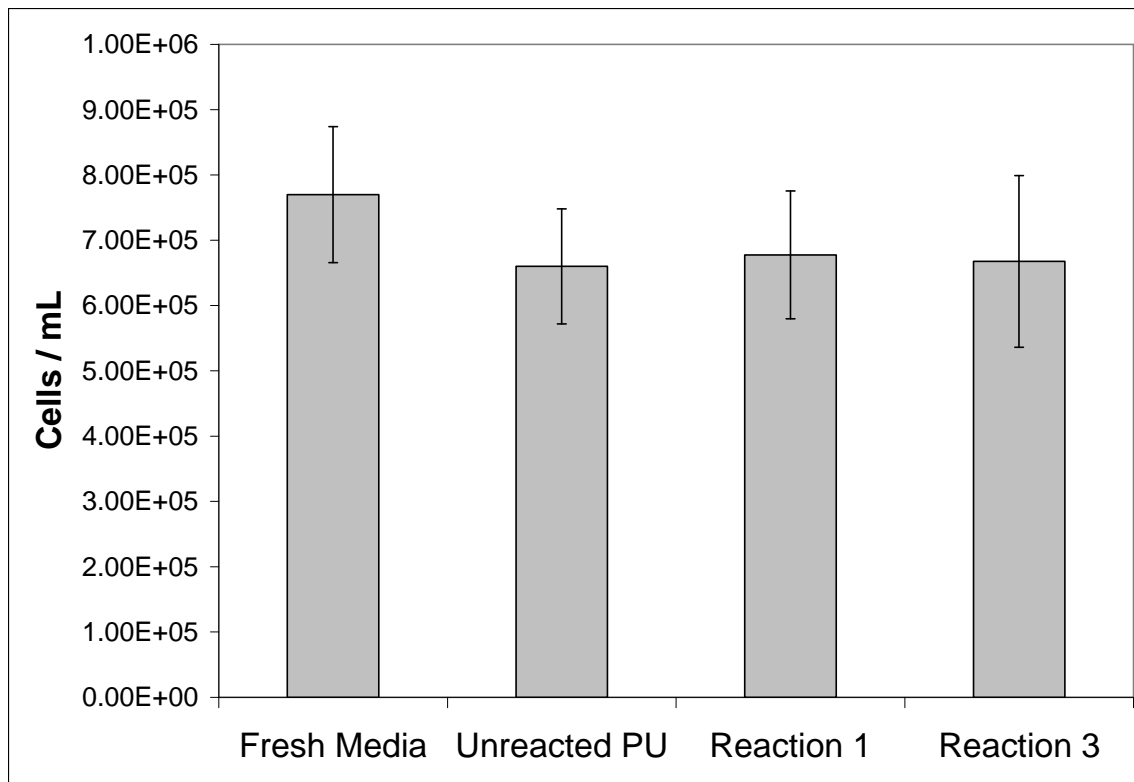


Figure 13. Leachables Analysis of Modified Polyurethanes

Cell count of fibroblasts following culture on tissue culture plates with DMEM media containing leachables extracted from unmodified polymer films and modified polyurethanes. No significant difference was observed between fibroblasts cultured in media incubated with unmodified PU and modified PU.

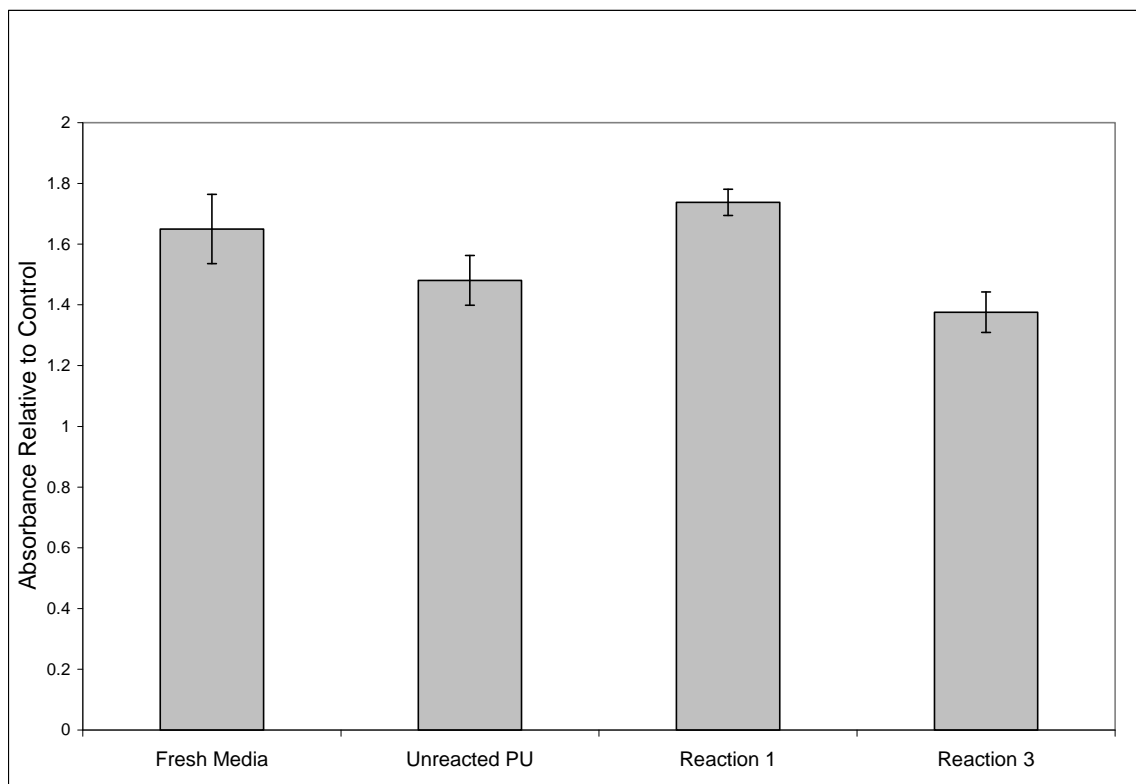


Figure 14. Cell Viability Assay

The viability of fibroblasts grown in media incubated with polyurethane samples. MTT assay demonstrated no significant difference in cells grown in media incubated with control PU and modified PU samples.

% Hemolysis	Average	Standard Deviation
Unmodified Polyurethane	1.32	0.333
Samples through Reaction 3	1.72	0.253

Figure 15. Hemolysis of Modified Polyurethane

Percent hemolysis of fresh whole blood following incubation with control Chronoflex AR and chemically modified PU samples. Five individual samples from 2 independent batches were tested and normalized to controls from their respective films.

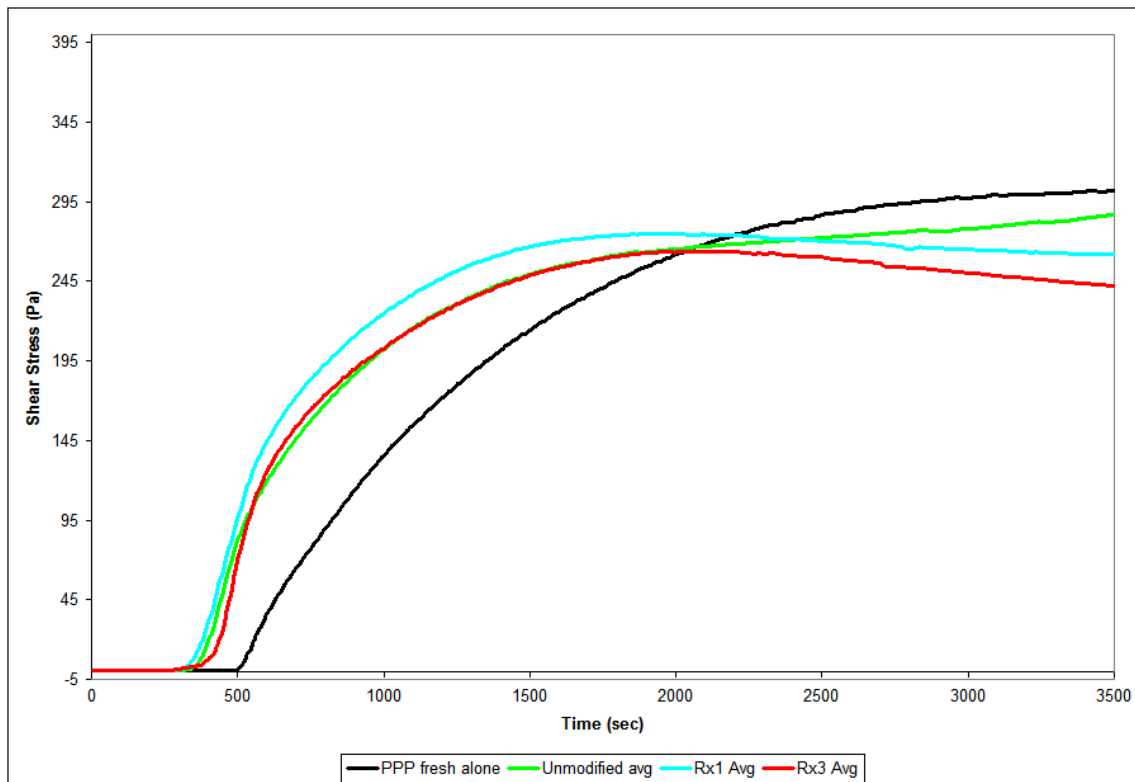


Figure 16. Thromboelastograph Analysis of Blood Compatibility of Polyurethane Samples.

Measure of the clotting characteristics of control Chronoflex AR and modified PU with platelet poor plasma by thromboelastograph. The chart tracks the time to clot initiation and the formation of the clot until it reaches its maximum amplitude.

Thromboelastograph Values					
		SP(sec)	R(sec)	K(sec)	MA(mm)
Unmodified	Average	350.0	388.3	220.0	34.6
	Std Deviation	31.2	34.0	70.9	3.6
Reaction 1	Average	346.7	381.7	148.3	35.4
	Std Deviation	63.3	63.3	35.5	2.9
Reaction 3	Average	326.7	408.3	185.0	33.9
	Std Deviation	52.5	20.2	65.4	1.0

Figure 17. Thromboelastograph Values

Measure of the clotting characteristics of control Chronoflex AR and modified PU with platelet poor plasma by thromboelastograph. No significant change was observed in the recorded times of initial fibrin formation (SP), time to clot initiation (R), the kinetics of clot formation (K) or the strength of the clot formed (MA) using PPP, between control PU and modified PU samples.

Proteins Attached	¹²⁵ I Labeled Protein		Bicinchoninic Acid	
	$\mu\text{g} / \text{cm}^2$	Standard Deviation	$\mu\text{g} / \text{cm}^2$	Standard Error
1 Protein via Acid	0.165	Calculated from Other Values	0.118	0.037
1 Protein via Oxirane	0.133	0.064	0.19	0.05
2 Proteins	0.298	0.037	0.242	0.041

Figure 18. Quantification of Protein Immobilized via the Bidentate Functional Group.

Quantification of protein immobilized onto each site of bidentate functional group.

Protein concentration of covalently bound protein was measured by the immobilization of ¹²⁵I labeled IgG and by reaction with bicinchoninic acid. Four individual samples from 3 independent experiments were tested and normalized to controls from their respective films.

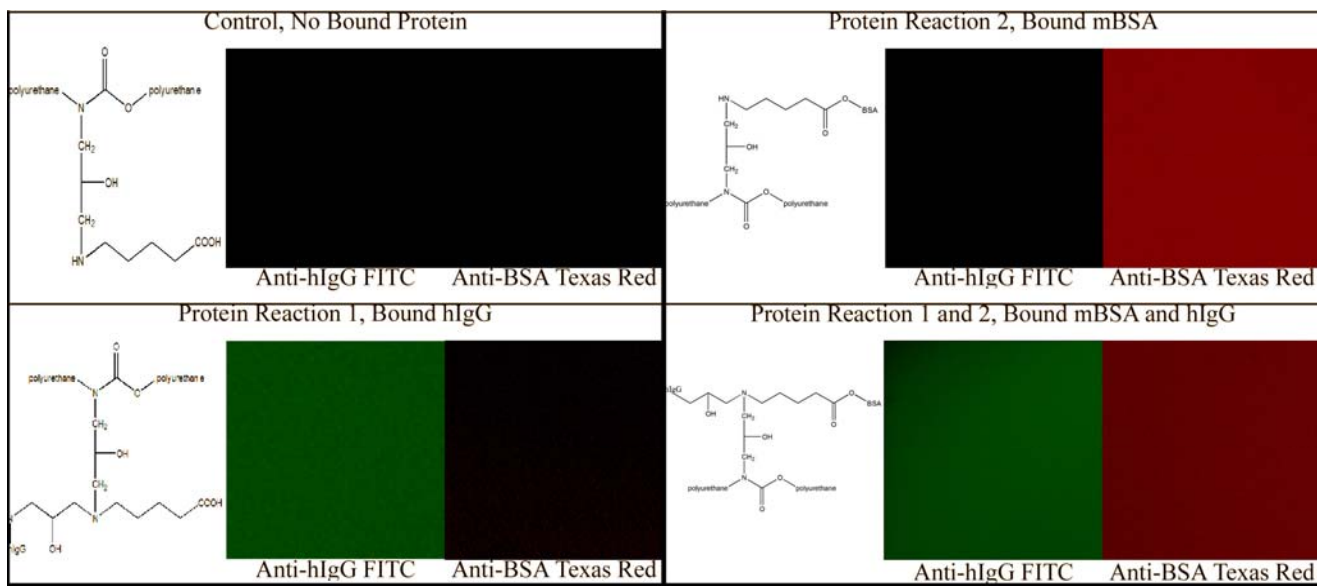


Figure 19. Qualitative analysis of protein immobilization via the bidentate functional group. Samples following protein immobilization, human IgG through the oxirane and BSA through the acid, were incubated with 1.5 mL of a 1:1000 dilution of anti-human IgG conjugated to FITC or a 1:100 dilution of anti-BSA conjugated to Texas Red fluorescent label overnight at 4°C. Samples were washed with 2 mL PBS + 0.05% Tween 20 for 30 minutes and repeated 3 times followed by washing with 2 mL PBS for 30 minutes and repeated 3 times to remove unbound antibodies. Surfaces were evaluated using a Leica DM IL Fluorescent microscope.

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Chapter 2

Mechanical Analysis of Polyurethane Modified for Sequential Protein Immobilization

Absrtact

Material elasticity and compliance have been studied for many years as they have been linked with the one of the main forms of failure, the formation of an intimal hyperplasia (IH). IH, a cellular formation composed of smooth muscle cells, is formed in areas where the blood flow, specifically the wall shear stress, has changed. Over time, IH found in vascular grafts has been linked to a mismatch of the material compliance of the synthetic graft to the native tissue causing an augmentation of the blood flow pattern.

Polyurethane (PU) is a polymer used in the making of experimental vascular grafts whose compliance and elasticity have been molded to exactly match that of a vessel. However PU's use in vascular system is limited by its blood compatability. This chapter will examine the mechanical properties of control PU and compare it to chemically modified PU developed in chapter 1 which will allow for the immobilization of proteins to increase the polymer's blood compatibility.

Introduction

Early design of vascular grafts were created from materials which were common and shown to be nontoxic such as polyesters (PE) and polytetrafluoroethylene (PTFE) with little regard to the mechanical properties of the material past long term durability, however a low 2 year patency rate was found for artificial grafts produced from these materials.[1] Over time it was discovered through explant studies that animal models implanted with these grafts showed an abnormal growth at the distal end of the graft site known as intimal hyperplasia (IH).[2-6]

The intima is the inner most of three layers which compose the blood vessel and is composed of the endothelial cell monolayer and a basement membrane formed by collagen (type IV), lamin and proteoglycans.[4, 7] Naturally as they age and incorporated in the diseases of hypertension and arthersclerosis there is a thickening of this layer created by the migration of vascular smooth muscle cells (VSMC) and production of their extracellular matrix by these cells. It is this growth which is referred to as IH. Abnormal IH formation is seen as a result of injury to the vessel, inflammation or an increased mean wall stress.[4] It is through an increased mean wall stress in which artificial grafts are believed to create IH.

Blood vessels are considered viscoelastic, meaning that they are not perfectly elastic, instead over time vessels will lose some of their mechanical properties due to stress relaxation and creep.[8-10] Like other viscoelastic materials, vessels do exhibit some elastic behavior which allows for expansion under systolic pressure. The degree to which they expand as well as the ability to contract back to their original are related to the mechanical properties of elastic modulus or stiffness and the compliance.[8, 9]

Modulus is the relationship of stress done on a material to the resulting strain of the material with the linearity of the response being related to the elasticity of material. The compliance is normally defined as the inverse of the elastic modulus of a sample, however for vascular mechanics this relationship is normally defined as the change in volume of the vessel to the change of pressure during a pulsed experiment.[8, 9] As a result of the expansion of the vessel under cardiac cycling, the modulus and the compliance affect both the flow rate and the flow geometry of the blood.[11] It is these factors which are believed to cause the change in the wall shear rate which leads to the development of IH.[4-6]

This phenomena, which is normally referred to as compliance mismatching[6, 12, 13], has lead researchers to change the internal diameter of the grafts to maintain the native flowrate. While this technique has corrected the linear flowrate and the average wall shear rate, it is not able to create the correct flow geometry.[14, 15] In addition the change in the internal diameter has lead to the occlusion from fibrous buildup for grafts where the diameter in decrease or the formation thrombo-emboli in grafts whose diameter was increased. Vein cuffing of polymer grafts is another method which has been investigated to try and correct the issue of compliance mismatch. In this case the vascular graft has a portion of harvested vein attached to the distal end of the graft. In doing this the vein which tends to be more compliant than arteries absorbs much of the pressure of the increased flow.[16-18] This method does not completely rectify the problem of flow geometry and also presents the issue of artificial vascular grafts being used largely for patients whose veins are damaged through disease that they are not suitable for transplantation. Other research has been conducted on the formation of grafts

using multilayer constructs.[19] By creating multilayer constructs, where the different layers have different mechanical properties, researchers have attempted to mimic the multiple phases of a blood vessel. These methods have included using a type of stent placed around a vein to restrict its expansion or the formation of layers with different porosities or crosslinking and have produced grafts of varying success.[20]

The most promising vascular grafts have been produced using polyurethane. Polyurethane being a copolymer can have many of its mechanical properties tailored to the biological and mechanical requirements. As a result polyurethanes naturally have mechanical properties on the same order as those of native arteries. These mechanical properties have been coupled with different molding techniques, such as the incorporation of ridges on the outside of the graft to limit expansion, to create grafts whose compliance is identical to that of the artery which is being replaced.[21-23]

While the initial compatibility of the vascular graft has been focused on in the design, the mechanical properties of polymer grafts also influence the long term durability of a potential implant. Until recently, artificial grafts have mainly been studied for 2 year patency, however this is due more to the development of IH or fibrous occlusion than actual failure of devices. In some cases it was found that certain polymers were susceptible to biodegradation, this was specifically the case of polyester urethane and polyurethane urea. Newer versions of polycarbonate urethanes have proved much more resistant to the hydrolysis which caused device failure.[21] Larger durability issues stem from the viscoelastic nature of polymers and the stress relaxation and creep phenomena which alter the mechanical properties over time and could eventually lead to a failure.[24] Polyurethane used in the construction of heart valves have shown the

ability to withstand 10 to 12 years worth of cycling in accelerated fatigue tests[25, 26] suggesting that should their blood compatibility be controlled, grafts constructed from these polymers could be suitable for long term implantation.

This study seeks to determine the effects on the long term durability of polyurethane which has been surface modified to improve upon its biocompatibility. In this method, polyurethane films were modified in the precut solid state form across the solid-liquid interface in an effort to reduce the effect on the bulk mechanical properties. The modified samples will be characterized by the initial modulus and by total stored energy of the polymer sample prior to and following fatigue cycling as a function of the solvent used during the modification. The effects of the fatigue on the mechanical compliance of the polymer samples were also studied do to the importance of compliance for developing vascular grafts. We predict that modified samples following fatigue will show no significant difference between modified samples and control polyurethane samples in either the stored energy or the compliance.

Methods

Chronoflex AR medical grade polyurethane was generously provided as a gift from Cardiotech Inc. (Cambridge, MA).

2.1 Preparation of Surface Bridge

Reaction 1 will enable the addition of the first reactive oxirane moiety to the secondary nitrogen incorporated in the carbamate subunit of the PU backbone. The addition of the reactive carboxylic acid branch of the bidentate bridge is achieved in reaction 2. Reaction 3 enables the addition of a reactive oxirane functional group to complete the bidentate bridge. Subsequent paragraphs will provide the experiment details of the surface modification.

2.1.1 Reaction 1

Solution cast polyurethane films were cut into 0.3 x 3.0 cm samples with an average weight of 30 mg. Under an inert atmosphere, approximately 1 gram of polyurethane was added to 75 mL tetrahydrofuran (THF, HPLC Grade 99.9%, Sigma Aldrich, St. Louis, MO) mixed with 75 mL hexanes (ACS Grade, Sigma Aldrich, St. Louis, MO) and the temperature of the mixture reduced to -5 °C using an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3

times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.2 Reaction 2

PU samples from reaction 1 were added to a 0.02 M 5-amino valeric acid (Sigma Aldrich, St. Louis, MO) in water. The samples were allowed to react at room temperature for 24 hours with stirring. Samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times. All absorbed water was removed by evaporation under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.3 Reaction 3

Under an inert atmosphere, PU samples from reaction 2 were added to a 150 ml mixture THF and hexanes (50/50 by volume) and the temperature of the mixture reduced to $-5\text{ }^{\circ}\text{C}$ in an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours. Samples were at room temperature until use.

Samples described in the above were prepared using a 50 / 50 by volume solvent mixture of THF and hexanes for reaction 1 and reaction 3, however similar reactions

were conducted using pure THF or pure hexanes as a solvent. Unless otherwise noted, all data described is for samples modified in a mixture of the two solvents.

2.2 Mechanical Analysis

2.2.1 Tensile Testing

Samples were cut to size and reacted as described above in THF, Hexane or a mixture of the two solvents (50% of each by volume). Samples were analyzed for stress at 37 C under a constant strain rate of 0.5 N/min to a strain of 5 N or failure using DMA Q800 (TA Instruments, New Castle, DE). The data was analyzed as stress as a function of strain. The initial elastic modulus was found by calculating the slope of the data over the initial linear region.

2.2.2 Energy Loss Fatigue Testing

Cut samples were prepared through reaction 3 as described above. Samples were mounted in tension in a DMA Q800 (TA Instruments, New Castle, DE) and equilibrated for 5 minutes at 37 C. Samples had their energy measured in an initial stress strain curve, 1-25% strain with a 5% strain/min rate and an unloading at the same rate. The samples were then fatigued from 1-12.5% strain at a strain rate of 250 %/min and unloading at the same rate for 500 cycles. Samples had their energy measured in a second stress strain curve, 1-25% strain with a 500 % strain/min rate and an unloading at the same rate. The samples were then fatigued from 1-25% strain at a strain rate of 500 %/min and unloading at the same rate for 500 cycles. Samples had their final energy measured in a

stress strain curve, 1-25% strain with a 5% strain/min rate and an unloading at the same rate.

Results

3.1 Tensile Testing

Samples were analyzed for stress at a linear strain rate and plotted as stress as a function of strain. The initial slope was calculated as the initial modulus and charted as a function of the solvent used during the modification of the samples and shown in figure-1. Surface modification PU without impacting the bulk properties was a desired research goal, as a change in bulk properties may manifest as a decrease in the material properties of PU. Thus as an initial step, reactions were conducted in THF, pure anhydrous hexane and 50:50 mixture of THF and hexane to investigate the effect (if any) of the solvent used on the mechanical properties of the material. As noted in figure-1, samples that had been reacted in THF had a significant change in the modulus in contrast to samples reacted in pure hexanes. This difference is attributed to the degree of absorption of the PU samples for the two solvent.

3.2 Energy Loss Fatigue Testing

Samples were analyzed for stress at a linear strain rate prior to and following two sets of mechanical cycling using a method modified from Beatty et al.[27]. The data was analyzed by integrating the area beneath the stress – strain curve, figure 2, to calculate the stored energy of the sample reported in figure 3. Despite the difference seen in the initial modulus in the prior experiment, there was no significant change in the stored energy prior to or following fatigue cycling for modified samples as compared to control samples.

During the same experiment the effect of mechanical cycling on the compliance of the polyurethane samples was studied. The data was plotted as compliance as a function of stress, figure 4-6, and the slope of the curve and minimum calculated, figure 7. Again, while significant differences were seen prior to and post fatigue, no significant change was found between samples which had been modified in either solvent and the control films provide from the manufacturer.

Discussion

The design of artificial small diameter vascular grafts has often focused on the blood compatibility of the materials used due to low patency rates as a result of occlusion. However, in vivo models have shown that the mechanical properties of vascular grafts also have a direct effect on graft patency through compliance mismatching leading to the development of IH.[11] Following this discovery of this relationship, several mechanical properties including elasticity, compliance and durability have been taken into consideration during design. Because of their natural compliance and biocompatibility, polyurethanes (PU) have become of interest for vascular implants. Methods have actually been designed to create vascular grafts with the exact compliance of the vessel they are replacing using Chronoflex PU, the same medical grade polymer used in this study.[21, 23] There is still a need to modify the surface properties of PUs to help prevent thrombosis and prevent occlusion of a potential graft, however it is also necessary for any modification to leave the bulk mechanical properties intact.

Following modification, PU samples were characterized by the initial modulus as a function of the solvent system used for modification. While a significant change was seen for samples modified in the pure THF or a 50/50 (v/v) mixture of THF and hexanes, the change in modulus was not great enough to prevent the use of the material in the design of vascular grafts as the modulus of the material is on the same order of magnitude as that measured in vascular tissues. Modification of the PU in pure hexanes did not show a change in the samples modulus as compared to control samples, however as was discussed in the previous chapter, use of THF aides in the swelling of samples during

modification and the removal of impurities from the surface of experimental samples left from initial processing.

The change in the initial modulus suggested a modification of the bulk properties, which could alter the long term durability of a created graft. To examine this possibility, the stored energy of the sample, as defined as the area beneath the stress strain curve, was measured prior to and following mechanical cycling in tensile mode. Stored energy is related to the ability of a viscoelastic material to recover following strain, similar to the energy in a spring.[8] As can be observed in figure 2 and figure 3, the stored energy of modified samples in either solvent system did not show a significant change from that of control PU samples. A significant change could be observed in each sample following cycling due to the plastic, non recoverable, changes to the PU samples. Modified samples also showed similar energies and curves following each step of the cycling which suggests that the samples did not lose their durability during the chemical modification and should fatigue in a manner similar to that seen in grafts constructed from unmodified PU.

Because of the importance of sample compliance to success of an implanted vascular graft, this property was also measure prior to and following mechanical cycling. Significant changes were seen between rate of change of the measured compliance and the minimum measured compliance of the samples following fatigue cycling, however as was observed in the stored energy, the compliance was not observed to be different in modified samples suggesting that these samples would function as samples designed from unmodified PU.

While this data has shown that there is no significant energy change or change in the compliance due to the surface modification, several outside factors also influence fatigue. All samples were measured at physiological temperature, 37°C, these samples were fatigued in a non wetted condition. Since part of the fatigue concerns have been associated with calcification[25, 28], it is unknown if the chemical modification will affect the absorption characteristics of the polymer, but it is reasonable to assume that since the modification completed on surface amines that the net ionic properties of the surface could be changed to affect long term durability. It is also important to note that these samples were analyzed in tensile mode while mechanical testing is normal designed to test the sample using a method which closely simulates that of natural use. Because samples were obtained as extruded films rather than in liquid solvent or molded in tube form which could be tested through pulsed forced flow, it was decided that stretching along the axis would be most representative of the forces. Finally it is also important to note that the force used for cycling was not physiological, but increased to do plastic deformations onto the samples in hopes that this would help to show what would occur in long term use rather than measuring under physiological conditions which would only demonstrate short term fatigue effects. However it should be noted for control PU and PU modified in both solvents all physiological loads, which is normally tested between 5 and 25 kPa,[10, 24] would be on the elastic portion of the their pre-fatigue stress strain curve (figure 2) and as such would have negligible energy loss.

Conclusions

Polyurethanes have been used in the design of vascular grafts because their mechanical properties, specifically the elastic modulus and the compliance, are on the same order of magnitude as vascular tissues. While methods have been designed to create small diameter grafts with the correct mechanical responses and flow patterns, there is a need to modify the surface to prevent fibrous build up which can lead to occlusion. This study has demonstrated that the modulus of the modified surfaces, while reduced is still sufficient for use in graft design. This study has also shown that the long term durability following mechanical cycling is not affected by the chemical modification.

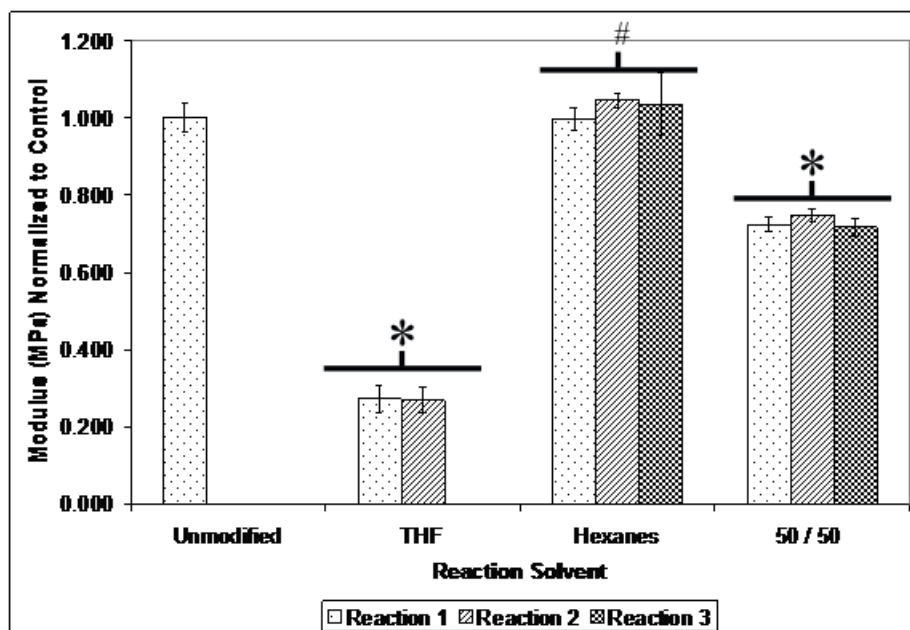


Figure 1. Mechanical analysis of Polyurethane Samples.

Initial modulus of control and modified polyurethanes as a function of solvent system used during modification. Modulus was determined in tension with a constant strain rate of 0.5 N / min at 37°C. Sample modulus was normalized to the average modulus of control unmodified Chronoflex AR. Average modulus of control, 8.695 ± 0.319 MPa. Error bars represent one standard deviation.

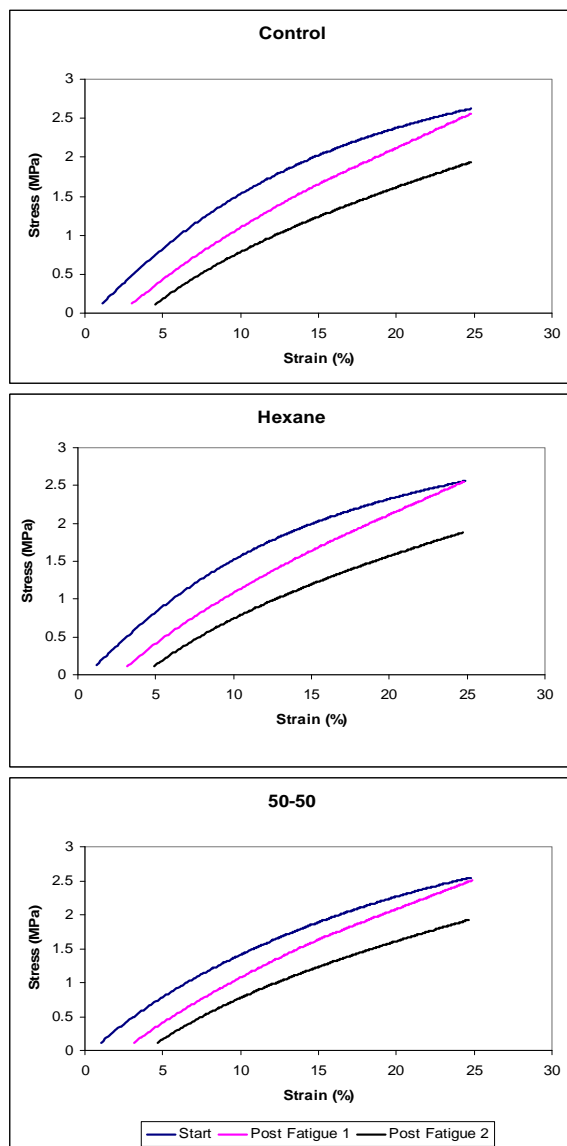


Figure 2 Stress Strain Curves as a Function of Solvent

Stress-strain curves were generated for control PU and modified PU samples prior to and following fatigue applications. Measurements were taken at a strain rate of 5% strain per minute until 25% strain was reached. Top curve in each panel taken prior to fatigue testing, middle curve in each panel taken after 500 cycles from 1 to 12.5% strain, bottom curve in each panel take following 500 cycles of 1 to 25%.

	Initial		Post Fatigue 1		Post Fatigue 2	
	Stored Energy MPa	Standard Deviation	Stored Energy MPa	Standard Deviation	Stored Energy MPa	Standard Deviation
Control	0.404	0.030	0.325	0.022	0.237	0.024
Hexanes	0.397	0.031	0.322	0.027	0.228	0.026
Hexanes / THF	0.382	0.007	0.318	0.005	0.235	0.010

Figure 3: Stored Energy of Polyurethane Samples Prior to and Following Fatigue Cycling

PU control and modified samples were measured to 25% strain at a 5% strain/min rate prior to and following fatigue cycling. The stress-strain curves were numerically integrated to calculate the stored energy of the polymer sample. No significant difference was seen in the stored energy between control PU and PU modified in either pure hexanes or 50/50 (v/v) mixture of hexanes and THF ($p > 0.15$)

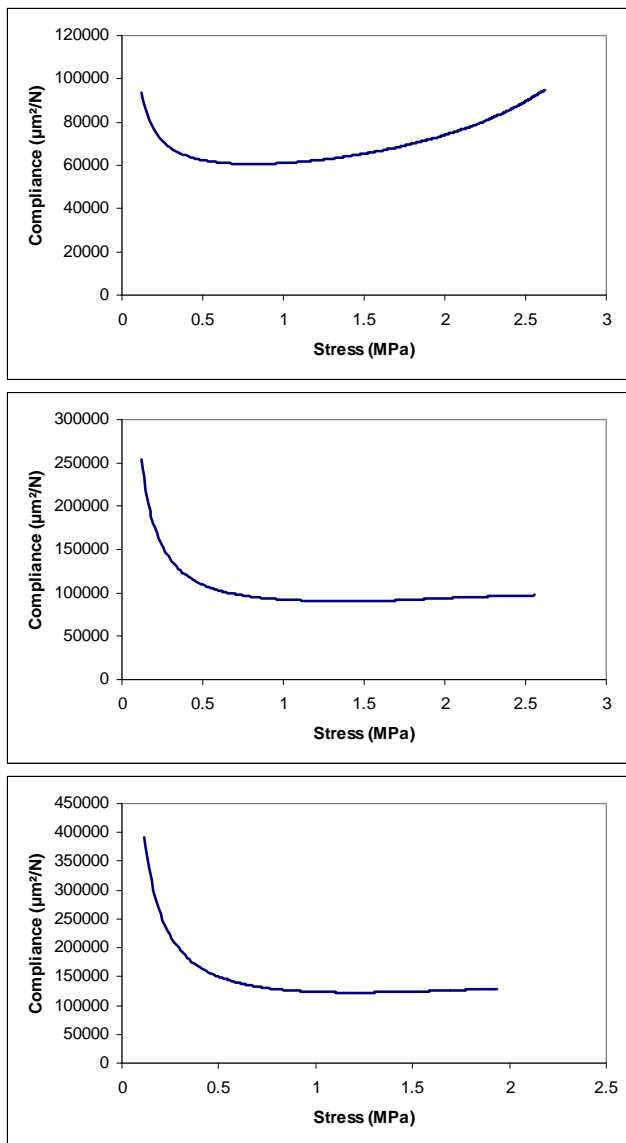


Figure 4: Compliance Curves for Control PU

Compliance curves for control Chronoflex AR PU prior to and following fatigue testing. Measurements were taken at a strain rate of 5% strain per minute until 25% strain was reached. Top panel taken prior to fatigue testing, middle panel taken after 500 cycles from 1 to 12.5% strain, bottom panel taken following 500 cycles of 1 to 25%. At least 5 samples composed of test strips from at least 2 solvent cast films.

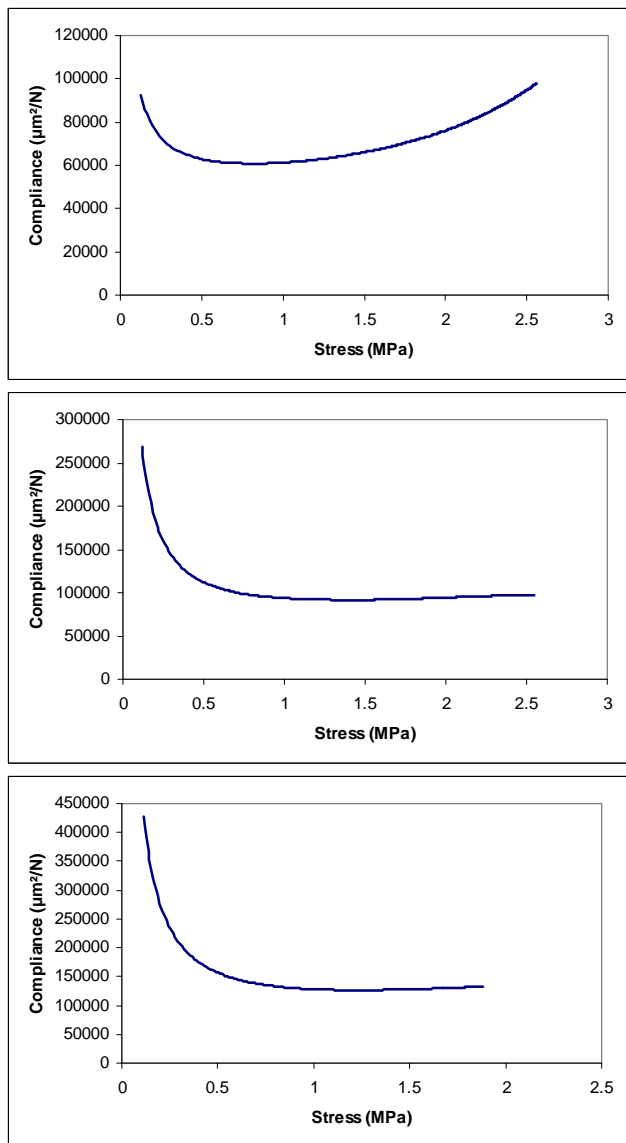


Figure 5 Compliance Curves for PU Modified in Pure Hexanes

Compliance curves for PU modified in pure hexanes solvent prior to and following fatigue testing. Measurements were taken at a strain rate of 5% strain per minute until 25% strain was reached. Top panel taken prior to fatigue testing, middle panel taken after 500 cycles from 1 to 12.5% strain, bottom panel take following 500 cycles of 1 to 25%. Data presented is composed of at least 5 samples composed of test strips from at least 2 synthesis.

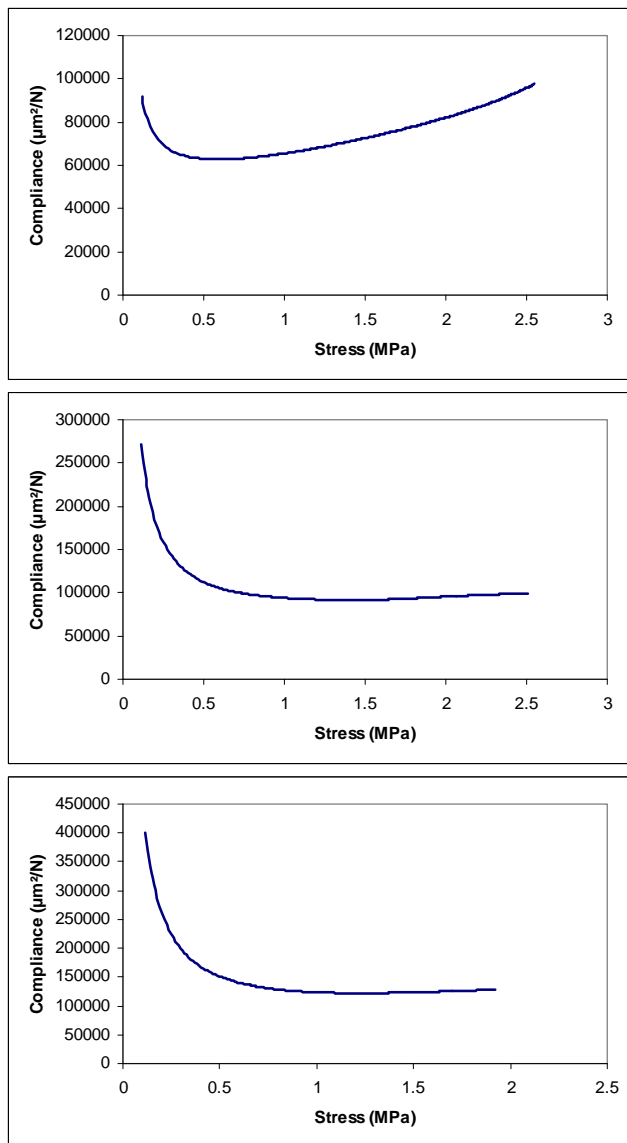


Figure 6: Compliance Curves for PU Modified in a 50/50 (v/v) Mixture of Hexanes and THF

Compliance curves for PU modified in a 50/50 solvent mixture of hexanes and THF prior to and following fatigue testing. Measurements were taken at a strain rate of 5% strain per minute until 25% strain was reached. Top panel taken prior to fatigue testing, middle panel taken after 500 cycles from 1 to 12.5% strain, bottom panel take following 500 cycles of 1 to 25%. Data presented is composed of at least 5 samples composed of test strips from at least 2 synthesis.

	Initial		Post Fatigue 1		Post Fatigue 2	
	Control	-1.63E+05	2.34E+04	-7.64E+05	7.02E+04	-1.27E+06
	Slope ($\mu\text{m}^2/\text{N}$)/MPa					
	Minimum ($\mu\text{m}^2/\text{N}$)	6.05E+04	9.02E+04	5.99E+03	1.22E+05	1.18E+04
Hexanes	-1.72E+05	4.16E+04	-8.49E+05	8.75E+04	-1.38E+06	1.58E+05
	Slope ($\mu\text{m}^2/\text{N}$)/MPa					
	Minimum ($\mu\text{m}^2/\text{N}$)	6.27E+04	9.15E+04	1.51E+03	1.22E+05	4.56E+03
Hexanes / THF	-1.63E+05	5.74E+04	-7.17E+05	2.32E+05	-1.50E+06	3.23E+05
	Slope ($\mu\text{m}^2/\text{N}$)/MPa					
	Minimum ($\mu\text{m}^2/\text{N}$)	6.07E+04	9.16E+04	8.20E+03	1.27E+05	1.37E+04

Figure 7 Summary of Compliance Testing

Slope and minimum reached of control Chronoflex AR PU and modified PU following fatigue testing. First column is value with second column be the standard deviation of at least 5 samples composed of test strips from at least 2 synthesis.

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Chapter 3

Development of an Endothelial Cell Mimicking Vascular Surface

by Co-immobilization of Thrombomodulin and EPCR

Abstract

In the normal endothelium; thrombomodulin (TM) and endothelial protein C receptor (EPCR), are well-integrated to regulate the protein C (PC) anticoagulant pathway. Recent studies have demonstrated that surfaced coatings of TM are able to prevent some of the complications involved with fibrous clot formation on natural or synthetic surfaces. However, at a certain surface density, the TM activation process becomes substrate transport limiting, a process which EPCR overcomes in vivo by increasing the surface transport of PC to the endothelial cell surface. The central goal of this study is to examine the influence of co-immobilizing rEPCR with TM on a biomaterial surfaces has on the activation of PC. TM and EPCR were immobilized on Ultrabind™ membranes, a porous surface which covalently binds proteins often used in immuno-blotting and biological sensor development. Membrane bound TM activated PC in increasing amounts until a surface density of 50-100 ngs was reached confirming previous data however coimmobilizations of TM and rEPCR were statistically indifferent from samples containing TM alone.

Introduction

Small diameter vascular grafts suffer from patency issues resulting from thrombi initiated occlusion. Biomaterial induced thrombosis is a complex issue, which involves several different enzymatic processes and is also impacted by the flow dynamics and the interactions of the material surface with blood or serum.[1-3] To better understand this process, it is necessary to review the systems involved in the mechanism of thrombosis, the methods that the body employs to maintain haemostasis and finally potential methods used to overcome thrombosis for artificial surfaces.

The primary mechanism of thrombosis, *in vivo*, is effected through the activation of the clotting cascade that relies on two separate pathways, the intrinsic and extrinsic pathways, which finally converge to common final pathway.[4] However on implants and biomaterial surfaces, the pathway is initiated by the adsorption of initiator proteins, prekallikrein, high molecular weight kininogen (HMWK) and Factor XII.[1, 2] The autoactivation of Factor XII to Factor XIIa is thought to begin the intrinsic clotting cascade, which in turn activates downstream targets, Factor XI and Factor IX. The extrinsic clotting cascade is activated through the protein tissue factor (TF). TF is a membrane bound protein found at the site of vascular injuries. In this system, Factor VII is absorbed onto the cell surface where it is activated by TF to form a TF/VIIa complex.[5] This complex and Factor IXa are both able to activate Factor X which is the beginning of the common pathway. Factor Xa interacts with Factor Va, Ca^{2+} , and phospholipids to form the prothrombinase complex which converts prothrombin to thrombin, the enzyme which polymerizes fibrinogen to fibrin, forming fibrous clots.[6]

Also involved in clot formation are platelets. Platelets are anuclear cells which circulate in the blood inertly until activated and are designed to give mechanical stability to the formed clot. The activation of platelets can come through several different mechanisms, however most of these mechanisms are protein receptor regulated.[7] Platelets are able to be activated by extracellular matrix proteins such as collagen and blood proteins which attach to the sites of vascular injury including fibrinogen and von Willebrand Factor (vWF) allowing for attachment at the site of vascular injury.[7, 8] Cell membrane proteins such as epinephrine, thromboxane and platelet activating factor can also activate platelets and proceed through a mechanism where these proteins are upregulated upon vascular cell injury or which are normally only found in sub-endothelial cells.[9] Upon activation, platelets elongate and release their granules which contain ADP which is able to activate more platelets as well as stimulating their aggregation.[10] Platelets are also able to be activated through thrombin allowing for a connection to the intrinsic pathway.[7] This process is further linked by the platelet acting as a site for Factor XIa and Factor IXa which then proceeds to the common pathway and further thrombin generation and fibrin polymerization.[4]

Thrombin is a key enzyme that links these two processes, creating the fibrous clot through the polymerization of fibrin, activating platelets and forming fibrin networks which stabilize platelet clots.[10-12] Thrombin is the active form of the zymogen prothrombin formed by two polypeptide chain linked through a disulfide bond. Thrombin exists in two forms, a fast form and a slow form which regulated through its binding with sodium. In the fast, more enzymatically active form, Na^+ ions are bound to a region with a high concentration of negatively charged amino acids near the C-terminus

of the enzyme.[13, 14] The binding of sodium creates a three dimensional change in the formation of the active site of the thrombin enzyme. These changes have been shown to change the substrate binding kinetics (K_m), the rate of reaction (K_{cat}) and the enzymes ability to react in the anticoagulant protein C pathway.[12]

Thrombin is very interesting within the coagulation cascade as it both up regulates and down regulates its own concentration within the clotting cascade. In the intrinsic pathway, thrombin activates Factor VIII which forms a complex with Factor IXa. The Factor VIIIa / Factor IXa complex has been shown through $10^5 - 10^6$ more active than Factor IXa alone and it is through this formed complex that greater than 90% of the Factor X used in a clotting reaction is activated.[15] Thrombin also activates Factor V, which then forms a complex with Factor Xa that results in thrombin formation. This complex has been shown to be 300,000 times more active than Factor Xa alone.[16, 17] The production of Factor Xa in the absence of Factor VIIIa and the production of thrombin in the absence of Factor Va is often considered a part of the initiation and the priming concentration of thrombin is necessary for an actual clotting event.[18] Thrombin also influences the down regulation of its concentration through the activation of the anticoagulant Protein C, one of the critical molecules to the regulation of haemostasis.[19]

Thrombosis on the surface of blood contacting biomaterials is a complex process that begins with the adsorption of serum proteins whose composition and rate of adsorption are partly dictated by surface characteristics.[1, 20, 21] The initial adsorption of proteins is based upon the concentration of the proteins in the blood, as a result initial adsorption has the greatest concentration of IgG, fibrinogen and albumin.[20] Adsorbed

albumin has been shown to passivate a surface to thrombosis, however adsorbed IgG has been shown to activate the complement system, which regulates inflammation.

Adsorption of fibrinogen onto an artificial surface induces a conformation change which exposes receptors for platelet binding.[22] Over time, high concentration but low molecular weight plasma proteins such as fibrinogen are exchanged for higher mass proteins such as HWMK in what is known as the Vroman Effect.[20, 23] As HWMK is able to activate the intrinsic clotting cascade, it is able to activate and recruit platelets to the fibrinogen remaining on the surface as well as form the fibrous network to stabilize the thrombus. Platelet adsorption onto biomaterial surface has been shown to be shear rate dependant.[24] At very low and high shear rates implanted surfaces have been shown to have low platelet adsorbance, however at intermediate flow rates platelet adsorbed at a high rate onto the biomaterials surface.[25, 26] Platelet adsorption has also been shown to be dependant on different adsorbed proteins based upon the wall shear rate of the flow.[8] At low and medium flow, in vitro tests have shown platelet adhesion to be fibrinogen dependant where as at higher shear rates, the adsorption is mediated by vWF which can also be adsorbed through the Vroman effect.[8]

The body and specifically the vascular endothelium provide several methods of regulating the different methods of thrombosis.[9] The first method of regulation is the vascular cell membrane which is able to inhibit the nonspecific adsorption of serum proteins, inhibiting fibrinogen, HWMK and vWF adsorption which causes biomaterial thrombosis. This phenomena is attributed to the chemical composition of the exposed membrane and the net negative charge of the membrane.[27]

The second method used by the endothelium to regulate clot formation is through the use of soluble molecules such as prostacyclin and nitric oxide which inhibit platelet activation. Prostacyclin is a lipid molecule produced in endothelial cells from the reaction of membrane phospholipids with cyclooxygenase-2 (COX-2) and prostacyclin synthetase.[9, 28] Once released from the cell, prostacyclin has two roles, when released into the smooth muscle of the vessel, the molecule causes vasorelaxation and when released into the blood reacts with platelets increasing the cAMP concentration which leads to decreased activation and aggregation.[29, 30] Nitric oxide (NO) is also synthesized in the cell and acts in a very similar manner to prostacyclin. NO is produced through the reaction of arginine and nitric oxide synthase. Once released, NO like prostacyclin reacts with platelets but produces cGMP, having a similar decrease in platelet activation and aggregation.[9, 31]

The final method of regulation of haemostasis by the vascular endothelium is through many membrane bound proteins and molecules including heparin and thrombomodulin, both of which inhibit thrombin formation and propagation. Heparin is a proteoglycan found on both the surface of the basement membrane of the sub-endothelial matrix and on the endothelial cell membrane.[9, 32] Heparin, both membrane bound and excreted, is able to bind a circulatory serpin, antithrombin III, producing a conformation change in the antithrombin III which increases the activity of the molecule to bind and inactivate thrombin and Factor Xa more than 100 fold.[33]

Thrombomodulin (TM) is a 60 kDa transmembrane glycoprotein which binds thrombin in a 1:1 ratio and acts as a cofactor for the activation of the anticoagulant Protein C.[34] TM is found on all vascular endothelial cells but its concentration is

variable with the largest surface density in capillaries.[35] TM binds thrombin in its fifth and sixth epithelial growth factor like domains, when bound the thrombin is unable to catalyze the formation of fibrin from fibrinogen, but also activates Protein C at a rate greater than 1000 faster than unbound thrombin.[12, 19, 36] Though the binding of thrombin is reversible and very fast with a half life of approximately three seconds[37], the binding of thrombin to TM has been shown to be make the thrombin more susceptible to antithrombin inactivation by greater than 20 times due to a chondroitin sulfate branch near the bound thrombin.[38] In addition, any active thrombin released by TM is in the slow conformational form. The importance of TM is evident in the study where a 30 fold loss of TM was seen in the first 3 days after implantation venous vascular graft associated with early graft failure normally from thrombus formation.[39]

Protein C (PC) is a 62 kDa zymogen which circulates through the blood at a concentration of 0.0645 μM , significantly less than the 2.4 μM of antithrombin III.[12] PC once activated by the thrombin /TM complex reacts through competitive inhibition with Factor Va and Factor VIIIa.[40] The inhibition of these molecules inactivates the Factor VIIIa / Factor IXa complex used to produce Factor Xa and the Factor Xa / Factor Va complex used to activate thrombin.

Though the concentration of PC is lower than that of antithrombin III, activated Protein C (aPC) plays a vital role in the regulation of haemostasis, both during clotting events and under normal flow.[35, 40] In the capillaries, the concentration of TM is large enough to bind all activated thrombin which passes through the capillary bed with the production of aPC in the capillaries able to prevent occlusion from fibrous polymerization.[41] In larger blood vessels, aPC is released into the boundary layer near

to the vascular wall. Due to its half life of approximately 15 minutes, the aPC unlike the antithrombin which must remain bound to heparin to remain active is able to be released into the blood and inactivate thrombin formation near or on the surface.[40]

The rate of activation by PC by TM can be increased by its co-localization with endothelial protein C receptor (EPCR). Values for the increase vary between 5x and 20x increase in the rate of reaction, however blocking the active site of EPCR in baboon arteries in vivo showed an 86% decrease in the production of aPC.[42] The concentration of EPCR is variable in the vascular system with high concentrations in large arteries and the umbilical cord with no EPCR found on endothelial cells of capillaries and reaching a ratio of approximately 7:1 for HUVEC cells.[43] The areas of largest concentration of EPCR are areas containing a 100 time increase in the blood to TM ratio over that of capillaries suggesting that EPCR is most necessary where PC in the blood might need to be transported to the TM protein for activation.[44] It is through this mechanism that EPCR functions. Studies showed that EPCR has an effective binding constant of 29 nM towards PC, in comparison to 266 nM when EPCR or Mg^{2+} , a necessary ion for function, is blocked.[45] Crystallography studies further confirmed the mechanism by showing that when bound to EPCR, the cleavage site for activation is presented outward for reaction with the TM/ thrombin complex.[35, 46] In addition studies attempting to incubate EPCR with TM were not able to isolate any EPCR / TM complexes suggesting that the two proteins do not interact physically.[45] This mechanism does have a limitation as EPCR shows the same binding efficiency for both PC and aPC. As a result of this, aPC can remain on the surface bound to EPCR and in this surface bound state shows less activity as an anticoagulant.[45] aPC bound to EPCR

has also been shown to be more stable and the overall efficiency of the TM/ thrombin activation system is not inhibited as it is when EPCR is fully blocked by antibodies.[47]

Due to the thrombogenic nature of biomaterial implants, several different methods used by the endothelium and nature have been incorporated into surfaces in an attempt to reduce clot formation. To reduce the adsorption of nonspecific proteins materials have been coated with molecules similar to or found in the cell membrane. Often phospholipids are used to create the coating[48, 49] which also allow for the anchoring of cell membrane proteins with trans-membrane domains to attached in a manner which orients them correctly and maintains their proper three dimensional structure.[49] Coatings of polymers, such as polyethylene oxide (PEO), polyethylene glycol (PEG), have also been designed to reduce the hydrophobicity of the surface of the material.[50] Other techniques to passivate the surface have been developed by attaching molecules from the blood. These molecules, which include albumin[51] and cholesterol,[52] create a surface coating which the body should not recognize as foreign.

Several surface modifications have been designed to control thrombin production on the surface of the material. These modifications range from the use of direct thrombin binding peptides to venoms to bioactive molecules found on the endothelial membrane.[53] Direct thrombin peptides are surface coatings which bind 1:1 with a thrombin molecule and block the active site of thrombin to prevent the formation of fibrin to fibrinogen. The advantage of this coating is that the peptides can remain stable for a longer period of time as compared to immobilized proteins because these peptides do not have a complex three dimensional structure. Hirudin is a specific example of a direct thrombin inhibiting peptide which has been isolated from the venom of leeches.[12] This

peptide has the ability to irreversibly inactivate thrombin and also has the ability to bind to and inactivate thrombin which has already bound to fibrin.[54] A limitation of hirudin and other direct thrombin inhibitors is the 1:1 ratio for inhibition and that these peptides do not deactivate the clotting cascade which continues to activate thrombin.

The immobilization of heparin has been investigated as a method of decreasing thrombosis on blood contacting devices for several decades and has been used as a coating for circulating devices, stents and vascular grafts. Heparin has been experimented with on several different materials including polyethylene[55], polyester[56], polyurethanes[57], expanded polytetrafluoroethylene[58] and collagens[59] covalently bound either directly to the surface or through a chain extension from the surface. In these cases, heparin immobilized surface have shown a lengthening of the clotting time. The ability of the heparin to inhibit the clotting mechanism has been shown to depend on the molecular weight of the heparins immobilized, the orientation of the heparin on the surface and whether a spacer arm was used to extend the heparin molecule off the material surface.[50] Generally, heparins immobilized through their reducing ends and extended from the surface by a spacing polymer are more active due to the better exposure of the active sites.[60, 61] Despite the immobilization techniques, the activity of heparin is significantly decreased by its immobilization onto the material surface, estimated to retain only 1% of its activity.[50] This loss of activity is partially expected as antithrombin III is only active when bound to thrombin and the complex is unable to inhibit thrombin that is already reacting with fibrinogen, as biomaterial surface bound thrombin would be. There is debate if the reduction of clotting is due to the formation of the heparin / antithrombin III complex and the thrombin inhibition

mechanism, instead suggesting that the heparin coating binds to other blood proteins with heparin binding sites creating a layer of specific blood proteins which acts in a manner similar to an albumin coating.[62] Despite the mechanism, heparin coated grafts have shown slightly better patency rates in one and two year trials,[63, 64] though in studies the ability to inhibit thrombin formation has been no different than control samples.[65]

TM has also been covalently immobilized onto several different surfaces including expanded polytetrafluoroethylene[66], polyurethane[67], polyacrylic acid[68], cellulose[69], agarose[70], nitinol[71] and glass[72]. Coatings of TM range in concentration from $1.5 \mu\text{g}/\text{cm}^2$ [71] to physiological levels found on the surface of the endothelial cell of approximately $10 \text{ ng}/\text{cm}^2$ [49] during which the activity of the coating was shown to be proportional to the concentration of TM until the rate reached a maximum at approximately 8x physiological levels.[49] Most samples have been tested to show the ability of the surface to activate PC in the presence of thrombin under static or flow conditions, however TM coated surfaces have also shown the ability to decrease platelet activation and adhesion in vitro.[71, 73] Clotting experiments on TM immobilized showed a significant extension to clot formation when compared to either control surfaces or surface coated with albumin. During these experiments, the activity of surface immobilized TM was compared to soluble TM which showed surface activity of 10-33% that of soluble TM.[67, 68] While this loss of activity is significant, it is less activity loss than the 1% of soluble activity seen with heparin coated surfaces. The activity of TM is a concern as immobilized proteins are susceptible to cleavage and can denature over time. Although long term data is not available, activity tests have been

conducted for 90 days, showing an initial activity loss during the first 15 days at which point a stable activity existed for the duration of the study.[49]

Recently, surfaces have been designed to better mimic the endothelium by combining several of the methods of regulating hemostasis found on the membrane. These surfaces combine the effects of phospholipid membrane with TM[49], a membrane and TM and heparin,[74] and TM and heparin and nitric oxide production[31]. The methods combine methods used to control thrombin concentration, prevent non-specific protein adsorption and prevent platelet adhesion and activation. The addition of lipid layer was designed to serve a second purpose ensuring the proper alignment of the TM on the materials surface increasing the activity.[49] The addition of heparin to TM surfaces would seem to create a competition for available thrombin to activate the TM / PC reaction, however in vivo thrombin attached to TM is more susceptible to anti-thrombin inactivation and the addition of the heparin to the surface ensures all released thrombin is inactivated.[74] Surfaces releasing NO have been prepared in two manners, either through time release loading[31] or through the immobilization of copper compounds which react with nitrosothiols which circulate in the blood.[75] These surfaces have shown the ability to inhibit platelet adhesion and activation and also the ability to activate PC, however clotting time data or the interaction of these surfaces with blood has not been published.[31]

The research into the creation of vascular biomimetic surfaces has demonstrated that the immobilization of endothelial cell groups such as TM and heparin onto a polymer surface are able to significantly inhibit clotting effects when contacted with blood. Of these molecules, TM shows the most promise for immobilization onto implantable

surfaces due to its demonstrated ability to prevent hyperplasia on polyester grafts and its ability to completely arrest thrombin formation when expressed at arterial concentrations on venous grafts. TM's ability to completely prevent the formation of thrombin has not been able to be transferred from natural endothelial cell covered venous grafts to polymer grafts as the polymer implants do not contain a cell membrane which aids in the inhibition of clotting. While other studies have tried to incorporate a phospholipid layer on the material surface, they have found that the TM containing surface becomes PC transport limited.

This research proposes the covalent co-immobilization of TM with its endothelium membrane cofactor EPCR. In this study, we will produce a recombinant protein containing the cytosolic domains of the EPCR protein in *Pichia Pastoris* yeast and describe the purification of this protein via a histidine tag incorporated in the recombinant sequence. We will demonstrate that the EPCR produced will bind PC and does not inhibit the activation of PC by the TM / Thr complex. We hypothesize that by co-immobilizing EPCR with TM on a solid support the ability of the immobilized TM to activate PC will be increased.

Methods

Construction of an EPCR expression plasmid for expression and secretion in *P. pastoris*

Construction and expression of EPCR-LL-His₆ [76]

In order to construct the recombinant EPCR–LL-Xa-His protein expression plasmid pPICZαA-EPCR-Lys₂-Xa-His₆ (Figure 1B) primer pairs were designed on the base of the human EPCR sequence (corresponding to the residues 1–194, mature protein numbering) and inserting the coding sequences for two Lys residues, a Factor Xa cleavage site and six His residues at the C-terminal of EPCR sequence. The inserts were prepared via PCR, and the respective sequences of the forward and reverse primers designed to construct pPICZαA- EPCR-His₆ were:

forward: 5- AT**CTCGAG**AAAAGATTTTGTAGCCAAGACGCCTC-3

reverse: 5-

TAGCGGCCGCTTAGTGATGGTGATGGTGATGTCTCCCTTCAATCTTCTTCGA
AGTGTAGGAGCG-3

The bold underlined nucleotide bases indicate restriction-enzyme digestion-sites for *Xho I* and *Not I*. After amplification, the PCR product was digested by *Xho I* and *Not I* for at least 3h at 37 °C and then directionally ligated with pPICZαA vector (Invitrogen) that was previously digested with both enzymes. The ligated plasmid was transformed into competent cells of *E. coli* strain TOPO 10 (Invitrogen) and then a positive clone was selected. The sequence of purified plasmid from TOPO 10 was confirmed by sequencing in order to verify that the ligations and the His-tagged EPCR insert were correctly in-frame. About 5 µg of purified plasmid that was previously digested with *Pme I* and

transformed into X-33 *P. pastoris* competent cells to express rEPCR-LL-Xa-His. The resulting clone was designated as X33-EPCR-LL-His. The expression of the EPCR-LL-Xa-His was performed according the procedure described above for the expression of rEPCR.

Purification of rEPCR-LL-Xa-His using Ni²⁺-IMAC column

The tagged rEPCR was desalted using Econo-Pac 10DG desalting column (Bio-Rad) and purified in a single step by metal-chelating affinity chromatography. The Ni²⁺-IMAC purification (from 12 mL medium using 1 mL resin) procedure was carried out at 4 °C according to the manufacturer's instructions and rEPCR-LL-Xa-His protein was first eluted with 1 M imidazole, pH 7.9 and then desalted and concentrated using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore). The final proteins were stored in PBS, pH 7.2.

Evaluation of EPCR and hPC binding on surface immobilized rEPCR

Purified rEPCR1LL-His protein was spotted onto dry UltrabindTM membranes, as separate experiments. 1 µl of protein solution was applied per spot. Protein concentrations ranged from 0.0625 to 0.5 mg/ml in PBS buffer (10 mM phosphate dibasic, 25 mM NaCl, pH 7.2). The spots were allowed to air dry and then the uncoupled reactive sites on the membranes were blocked with 1M ethanolamine, pH 9.2 solution for 3 h at room temperature. The membranes were then separated into three sets. The first set was equilibrated with a 5 µg/ml of hPC solution in PBS, for 3 h at room temperature. Membranes were washed copiously with PBS and incubated with 1:1000th diluted HRP-

conjugated rabbit anti-hPC antibody for 3 h at room temperature. The second set was incubated with a 1:1000 dilution of anti-EPCR polyclonal antibody. Following washing with copious amounts of PBS, the membranes were incubated with a 1:1000 diluted HRP conjugated anti-rabbit antibody grown in goat. The third set of samples were incubated with a 5 µg/ml monoclonal antibody to EPCR designated RCR-2. Following washing with copious amounts of PBS, the membranes were incubated with a 1:1000 diluted HRP conjugated anti-rat antibody grown in rabbit. At the end of the incubation, membranes were washed and bound complex was detected via chemiluminiscent detection.

Solution Phase Protein C Activation Assays

Protein solutions of recombinant EPCR-his, human thrombomodulin (TM, American Diagnostica Inc., Stamford, CT), human thrombin (Thr, American Diagnostica Inc., Stamford, CT), human Protein C (hPC, Enzyme Research Labs, South Bend, IN), human activated Protein C (aPC, Enzyme Research Labs, South Bend, IN), were prepared in reaction buffer composed of 0.1 M NaCl (Sigma Aldrich, St. Louis, MO), 0.003 M CaCl₂ (Sigma Aldrich, St. Louis, MO), 0.0006 M MgCl₂ (Sigma Aldrich, St. Louis, MO), 0.02 M Tris-HCl (Fisher Scientific, Pittsburgh, PA), 0.1 mg/ mL Bovine Serum Albumin (BSA, Sigma Aldrich, St. Louis, MO) and the pH adjusted to 7.5. 25 µl of a 0.36 mg / ml EPCR-his was incubated for 15 minutes with 25 µl of a 0.1 mg / ml hPC solution in a sterile 96 well cell culture plate (Fisher Scientific, Pittsburgh, PA) Following incubation, 25 µl of a 0.006 mg/ml TM solution was added to the reaction mixture. 25 µl 0.06 µg / ml Thr followed immediately by the addition of Spectrozyme aPC (American Diagnostica Inc., Stamford, CT) to a final concentration of 0.0004 M.

The reaction was incubated at 37 °C with the absorbance at 405 nm⁻¹ taken after 0, 5, 10, 15, 20, 30, 45, 60, 75, 90 and 105 minutes using a Beckman Coulter AD 340 UV / Vis microplate reader. Samples were performed in triplicate and for samples without either EPCR or TM, the solution was replaced 0.08 mg / ml BSA solution. The concentration of Protein C activated was calculated using an aPC in reaction buffer calibration curve on the same cell culture plate.

Solid Phase Sample Preparation

Samples were prepared on 5 mm diameter punches of Ultrabind membrane made with a standard biopsy punch. Samples were created with varying concentrations of TM alone, TM and EPCR-his, EPCR-his alone, and TM and RCR2 – EPCR-his. EPCR containing samples were spotted in a 7:1 EPCR:TM concentration similar to that seen in vivo. Samples were spotted as described above in 25 µl drops in a 96 well cell culture plate and allowed bind for 2 hours followed by blocking with ethanol amine as described above for 1 hour. RCR-2 was immobilized at a molar ratio of 3.5:1 to TM to maintain the molar ratio of EPCR as each RCR-2 antibody can bind two EPCR molecules. Following blocking, sample containing were RCR2 were incubated for 5 hours with a 10x excess solution of EPCR-his, washed and cross-linked for 2 hours with 2% gluteraldehyde. Solid state samples were created at TM concentration of 0, 5 ngs/cm² (70 fmol/cm²), 10 ngs/cm² (140 fmol/cm²), 50 ngs/cm² (700 fmol/cm²), 100 ngs/cm² (1400 fmol/cm²) and 200 ngs/cm² (2800 fmol/cm²). All samples were washed and stored at 4 °C in reaction buffer.

Solid Phase Protein C Activation Assays

Samples prepared previously were washed in 50 μ l fresh reaction buffer prior to reaction. Samples were incubated for 30 minutes at 37 °C with 50 μ l of a 5 units / ml Thr solution with mixing. Following incubation, the thrombin solution was removed but the samples not washed. 50 μ l of a 0.025 mg / ml hPC solution was added to each sample and reacted at 37 °C with mixing for 1.5 hours. The reaction was ended by the addition of 10 μ l of anti-thrombin III (American Diagnostica Inc., Stamford, CT) to a final concentration of 6 units / ml. Following 15 minutes of reaction of anti-thrombin with thrombin from the reaction, the solution was pipetted from the solid samples to new 96 well plate. To each solution sample, 40 μ l of Spectrozyme aPC solution was added to a final concentration of 0.004 M. Solution samples were incubated at 37°C analyzed 405 nm^{-1} .

Results

Expression and Purification of rEPCR in *P. pastoris*.

In order to confirm the expression of rEPCR by *P. pastoris*, samples of yeast culture supernatants were electrophoresed and electroblotted using antibodies specific for EPCR. The expression level of rEPCR was estimated by western blotting to be about 20 mg/L of cell medium. The expressed rEPCR was heterogeneous, perhaps due to the glycosylation by the enzymatic machinery present in yeast, with three distinct protein bands which cross reacted with anti-EPCR polyclonal antibody (the first upper strong band, middle band and the third, weak band).

rEPCR-LL-Xa-His was purified from yeast culture medium (Figure 1B) to homogeneity in one step using metal-chelating affinity chromatography using Ni²⁺-IMAC column followed by ultrafiltration. The purity of rEPCR-LL-Xa-His was confirmed by SDS-PAGE in which proteins in the gel were first visualized by Coomassie Brilliant Blue followed by silver staining (Figure 1C). The purified protein appeared as a ~40 kDa polypeptide on SDS-PAGE, similar to rEPCR (without His tag) immunoprecipitated from HUVEC and EPCR-positive E-7 cells.[45] The expression level of rEPCR-LL-Xa-His was estimated to be ~6 mg per L of yeast culture medium.

Solution Phase Testing

The ability of TM to catalyze the activation of PC incubated with or in the absence of rEPCR was determined in solution. This experiment was designed to ascertain that rEPCR when incubated with hPC in solution would not impact the rate of activation of hPC by TM in the presence of thrombin. For samples lacking EPCR, a similar

concentration of BSA was incubated with the hPC. This experiment was also conducted in the absence of TM and in the absence of both rEPCR and TM to establish that rEPCR did not have any catalytic activity, and that the activation of hPC was not catalyzed by thrombin alone which is known to occur *in vivo* but a significantly slower rate. The influence of rEPCR in solution on Protein C activation is presented in Figure 3. The presence of rEPCR in solution did not impact the PC activation by the TR:TM complex. Solution phase analysis also showed that there was no activation of hPC in the absence of TM.

Dot Blot Analysis

Purified rEPCR was spotted on dry UltrabindTM membranes, and these studies were performed such that identical sets of replicate applications of immobilized rEPCR could be subjected to an ordinary sandwich ELISA to measure the immunosorptive capacity for hPC by surface immobilized EPCR or antigenicity and functionality could be confirmed by dot-blot analyses by reacting these membranes with monoclonal and polyclonal antibodies with documented reactivities to native EPCR. UltrabindTM membranes provide an activated surface for the covalent attachment of proteins and diffuse-reflectance-densitometry provides a precise detection of chromophoric signals and has been reported elsewhere.[77] The signal to noise ratio was greater than 100/1. A 2 to 4% variance in ELISA signals was found between replicate applications within a sheet. When hPC was not included in the ELISA procedures, essentially no signal was generated (data not shown).

UltrabindTM surfaces with immobilized rEPCR.

UltrabindTM membranes with indicated amounts of TM, rEPCR and rEPCR/TM were prepared as detailed earlier and the activation of PC by surface immobilized TM, rEPCR and rEPCR/TM was evaluated and presented in Figure 4. Controls correspond to membranes with no immobilized protein, however blocked and treated similarly as the test surfaces. Solid state samples were created at TM concentration of 0, 5 ngs/cm² (70 fmol/cm²), 10 ngs/cm² (140 fmol/cm²), 50 ngs/cm² (700 fmol/cm²), 100 ngs/cm² (1400 fmol/cm²) and 200 ngs/cm² (2800 fmol/cm²). Samples containing EPCR were immobilized at a molar ratio of 7:1 to mimic that found in vivo.[43] The increase in the amount of APC released correlated with increasing amounts of TM on the membrane until a maximum was reached between a surface concentration of 50 ngs of TM/cm² (700 fmol/cm²) and 100 ngs/cm². Co-immobilizations of EPCR and TM, albeit randomly, also yielded higher amounts of APC with increasing ratios of EPCR/TM until a maximum was reached at the same concentration of TM. While the concentration of APC was statistically different at the different surface densities of TM until the concentration of 50 ng/cm² was reached, samples immobilized with and without rEPCR with corresponding concentrations of TM were statistically indifferent from each other.

EPCR is functionally polar, in the sense that the hPC binding domain on the EPCR molecule resides on the –NH₂ terminal. To achieve directional immobilization, in separate experiments, RCR-2, a Mab directed against EPCR and that binds EPCR via a domain close to the –COOH terminal was first co-immobilized with TM at indicated

concentration and ratios.[78] RCR-2 was immobilized at a molar ratio of 3.5:1 to TM to maintain the molar ratio of EPCR as each RCR-2 antibody can bind two EPCR molecules. Upon completion of the protein immobilization step, the residual active sites were blocked and membranes were exposed to rEPCR solutions, washed and the rEPCR bound to RCR-2 was crosslinked with glutaraldehyde. The activation of PC on test surfaces with co-immobilized TM and RCR-2/EPCR were also evaluated and presented in Figure 4. While increased APC was noted at higher ratios, the differences were not statistically significant.

Discussion

Thrombomodulin (TM) is a surface bound protein whose concentration and activity has been shown to be relevant to the success of implanted venous vascular grafts.[39] In these studies veins implanted as arterial grafts showed a loss of functional TM which corresponded to a loss of patency due to thrombosis. Reintroduction of TM to arterial levels through gene therapy showed an increase in patency but was not able to overcome hyperplasia formation due to compliance mismatch of venous tissue. However these results suggest the importance of the TM / Protein C (PC) activation system to the regulation of thrombosis in potential vascular grafts. In other studies, TM immobilization has been shown to inhibit the formation of hyperplasia, a fibrous occlusion found on grafts with a compliance mismatch leading in to areas of improper blood flow, often found on vascular grafts constructed polyester.[79] Because of this importance, TM has been immobilized onto the several different biomaterials to test the ability of the immobilized protein to activate PC in both static and flow studies. In these studies, the activation of PC has been shown to be proportional to the surface concentration of TM until it reaches a concentration corresponding to approximately 8 times the native arterial concentration.[47] Native arteries however contain a surface bound cofactor to TM, Endothelial Protein C Receptor (EPCR) which shows a strong affinity for the PC increasing the activity of the TM mediated PC activation.[45]

Recombinant EPCR constructs were prepared in *Picia Pastoris* and designed with a secretion signal and a poly-histidine tail within the construct to create a one step purification process. The construct encoded the cytosolic portion of the transmembrane EPCR protein and was recognized by both polyclonal and monoclonal antibodies against

native EPCR as well as showing an activity towards the binding of HRP conjugated PC (figure 2). *Pichia Pastoris* was chosen to produce the recombinant EPCR due to its ability to produce recombinant proteins at higher fermentation rates than mammalian or plant cultured cells while enabling for secreted proteins with folding more similar to eukaryotic cells than those produced by bacteria due to its ability to correctly glycosylate asparagine amides and serine and threonine hydroxyl groups.[80] It is important to note that *Pichia* glycosylations contain different sugars, branching and length than proteins produced by mammalian cells which can produce an immune response, however *Pichia* has been shown to produce shorter polysaccharides than other yeasts, such as *Saccharomyces cerevisiae*. [81] More recently certain *Pichia* clones have been engineered to produce fully human glycosylated proteins which could be cloned into prior to in vivo testing.[82]

The binding of the EPCR by the monoclonal antibody designated RCR-2 served two purposes, first to identify the recombinant protein and secondly to create a method for proper oriented immobilization of EPCR. RCR-2 has been shown previously to identify a region near the transmembrane domain on the cytosolic EPCR and not alter the PC binding affinity to EPCR while bound.[78] By immobilizing RCR-2 onto a surface and then binding the EPCR through the antibody, it would ensure that EPCR would be extended from the surface in a manner mimicking the orientation from the cell membrane which should provide proper binding of PC.

Solution phase experiments were conducted to verify that the rEPCR protein will not inhibit or interfere with the ability of TM to bind PC that had been bound to EPCR as

well as verify that EPCR had no catalytic activity to activate PC in the absence of TM and TR. Upon analysis of these reactions it was determined that the incubation of PC with soluble EPCR did not affect the reaction rate of TM, figure 3. In addition, EPCR with a BSA replacement for TM showed no activation. These two results when combined confirm that EPCR does not catalytically activate the PC and does not create a conformational change in either the PC or TM to increase the activity, as is the case with other proteins involved with haemostasis such as the relationship between heparin and anti-thrombin III or thrombin and sodium. Instead, this suggests that the activation increase is likely caused by an increase in the transport of PC to the surface and proper presentation of PC by EPCR to TM on the surface.

Co-immobilizations were conducted using UltrabindTM, a preactivated membrane that enables the primary amines on proteins to react with the pendant aldehyde group on the membrane to yield covalent linkages via the formation of an amide bond, as a model surface. UltrabindTM is an ideal surface for initial testing due to its large protein binding and retention rates (306 $\mu\text{g}/\text{cm}^2$ when binding IgG with 89% retention following washes including detergent)[83] and because of these properties has been used in the initial phases of sensor development.[84] The limitation of immobilizations on this surface is that co-immobilizations can only be effected via differential reactivities of proteins or concentration gradients. While rEPCR and TM were both immobilized onto the same UltrabindTM membrane, the random nature could not ensure proximal immobilizations so as to catalyze the PC activation was not achieved. As a result no statistical differences were noted between the amount of APC generated by surfaces displaying TM or

TM+rEPCR. The orientation of EPCR did not affect the activation of PC on a randomly immobilized surface as there was no significant difference between samples containing TM and (TM+RCR2)+EPCR. The model did allow us to study the effect of TM concentration on the activation of PC and determine the concentration at which the activation reaches steady state, which confirmed similar studies.[49]

As the addition of the EPCR directly to the surface or via the RCR-2 showed no significant change, it must be determined why no increase was seen despite an expected 5x – 20x increase in the activity.[85] This lack of increase in activity could be due to the EPCR recombinant protein being inactive, the surface bound protein being inactive in the bound form or that the EPCR and TM are not immobilized on a scale to allow them to interact. Because EPCR attached to the ultrabind membrane was able to bind PC in the absence of TM shown in figure 2, it does not follow that EPCR is inactive. Also if there had been any loss in activity due to the method of EPCR binding to the surface, this should have been solved by first immobilizing the RCR-2 antibody onto the membrane followed by EPCR.

The mechanism by which EPCR acts upon the TM/PC is known to be related to the transport of PC to the endothelial surface from the blood with PC first binding to EPCR. Crystallographic studies of Protein C bound to EPCR show that when bound Protein C is presented in an orientation that allows it to be activated by the thrombomodulin-thrombin complex suggesting that Protein C is activated while still bound to EPCR.[46] This mechanism has been further confirmed by incubating Protein C with and without soluble EPCR prior to activating in solution phase, both having the

same reaction rates. Should this mechanism hold true, it would require that the EPCR and thrombomodulin be localized together on the surface. On the cell membrane, Protein C bound to EPCR has been determined through fluorescence energy transfer to be at a height of approximately 94 Å and thrombin bound to thrombomodulin at approximately 64 Å above the cell membrane.[86] If these protein complexes were approximated as a sphere with the height as the diameter where the reaction sites are able to move along the surface of the sphere this would require that EPCR and thrombomodulin be immobilized on a surface by a separation of less than 80 Å or 8 nm. While the 7:1 EPCR to TM ratio should produce so EPCR within this therapeutic distance, it was not enough to create a significant change in the amount of aPC generated in static experiments over samples with TM alone.

Conclusions

We have demonstrated a method for producing, purifying and characterizing recombinant human EPCR from *Pichia Pastoris* yeast. We have shown that these proteins can be immobilized on to surfaces and are active in their ability to bind PC. We have further shown that our recombinant EPCR does not inhibit the activation of PC by the TM / thrombin complex. Finally we have produced results which show that either an oriented or non-oriented state that EPCR must be colocalized within a specific 3D distance to TM to produce an increase in PC activation activity.

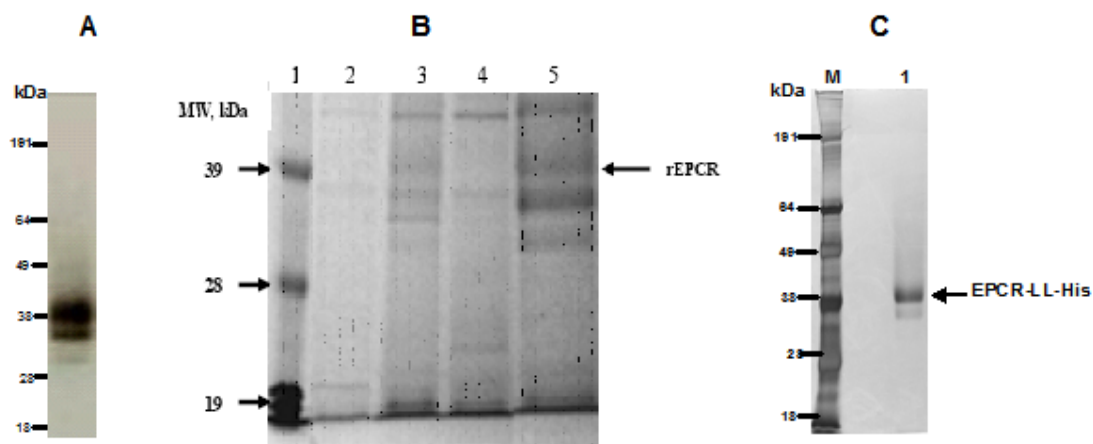


Figure 1: Analysis of recombinant EPCR production and purification.

Analysis of rEPCR produced as an excreted protein in *Pichia Pastoris* and purification by Ni^{2+} -IMAC column. A) Western Blot of media containing excreted rEPCR protein prior to purification. B) Commassie Blue stained SDS-Page electrophoresis gel for media containing rEPCR produced in *Pichia Pastoris*. Lane 1 positive control, lane 2,4 negative control, lane 3,5 EPCR expressing cells. C) Commassie Blue stained SDS-Page electrophoresis gel of rEPCR purification by Ni^{2+} -IMAC column followed by ultrafiltration.

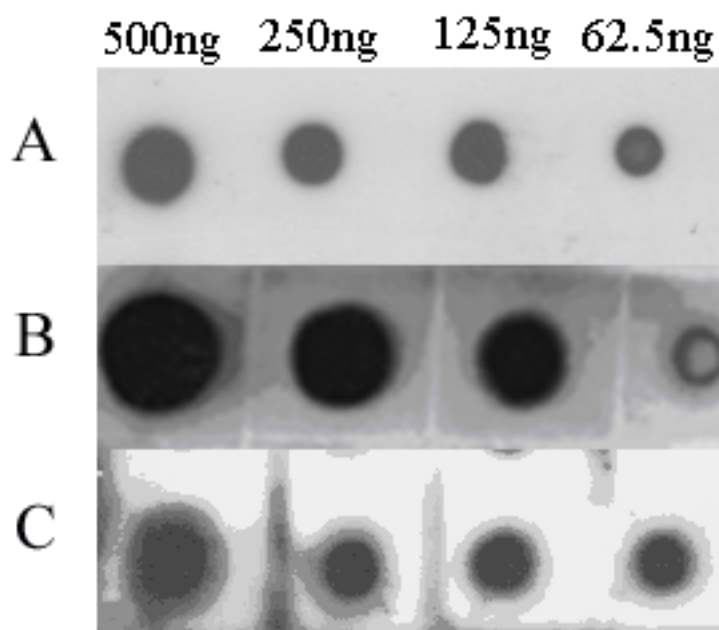


Figure 2: Characterization of EPCR.

Purified rEPCR protein was spotted onto dry Ultrabind™ membranes, as separate experiments. 1 μ l of protein solution was applied per spot. Protein concentrations ranged from 0.0625 to 0.5 mg/ml in PBS buffer (10 mM phosphate dibasic, 25 mM NaCl, pH 7.2). The spots were allowed to air dry and the reactive sites were blocked with 1M ethanolamine and surface-immobilized EPCR was detected as follows: A) EPCR antigen was detected using rabbit polyclonal antibody against anti-EPCR and HRP conjugated goat anti-rabbit antibodies as primary and secondary antibody, respectively, B) the presence of binding epitope was detected using RCR-2, a monoclonal antibody against anti-EPCR and HRP conjugated goat anti-mouse antibodies as primary and secondary antibody, respectively, and C) functionality (i.e. PC binding) was detected using pure human Protein C and HRP-conjugated rabbit anti-Protein C antibodies. Proteins were visualized with chemiluminiscent detection and quantified using Image software.

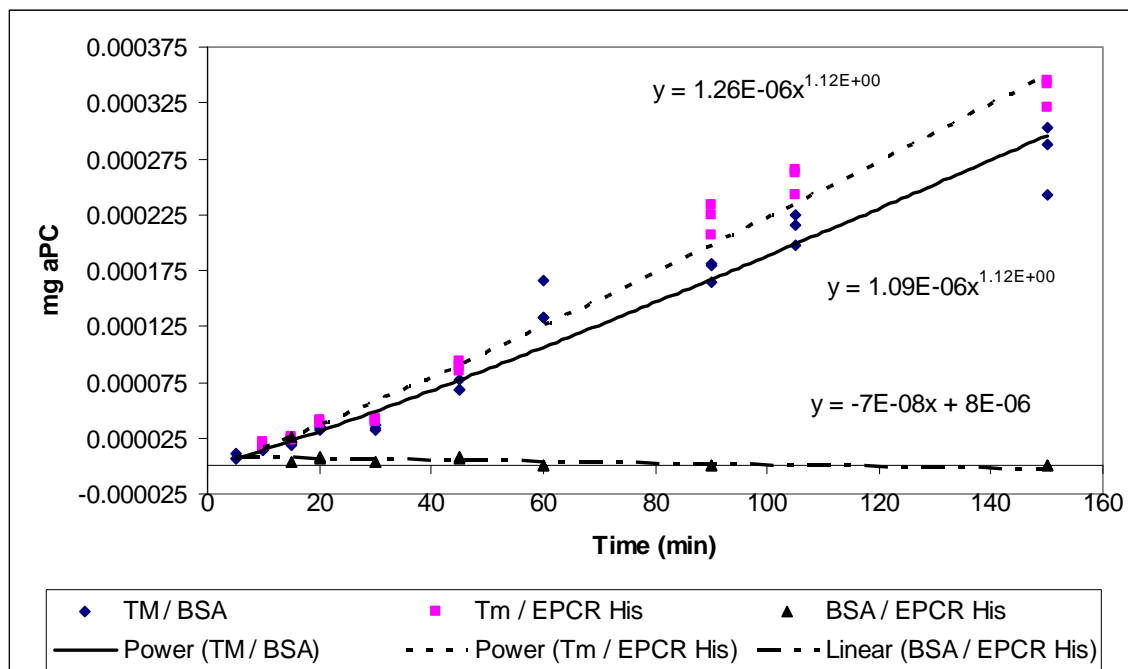


Figure 3: Solution phase activation of Protein C in the presence and absence of EPCR

Protein solutions of rEPCR, hTM, human thrombin, human PC, human APC were prepared in reaction buffer composed of 0.1 M NaCl, 0.003 M CaCl₂, 0.0006 M MgCl₂, 0.02 M Tris-HCl, 0.1 mg/mL BSA at pH 7.5. 25 µl of a 0.36 mg/ml rEPCR was incubated for 15 minutes with 25 µl of a 0.1 mg/ml hPC solution in a sterile 96 well cell culture plate. Following incubation, 25 µl of a 0.006 mg/ml TM solution and 25 µl 0.06 µg/ml of human thrombin was added to the reaction mixture and Spectrozyme aPC was added to a final concentration of 0.0004 M. The reaction was incubated at 37 °C with the absorbance at 405 nm⁻¹ taken after 0, 5, 10, 15, 20, 30, 45, 60, 75, 90 and 105 minutes using a Beckman Coulter AD 340 UV / Vis microplate reader. Samples were performed in triplicate and for samples without either EPCR or TM, the solution was replaced 0.08 mg/ml BSA solution. The concentration of Protein C activated was calculated using an aPC in reaction buffer calibration curve on the same plate.

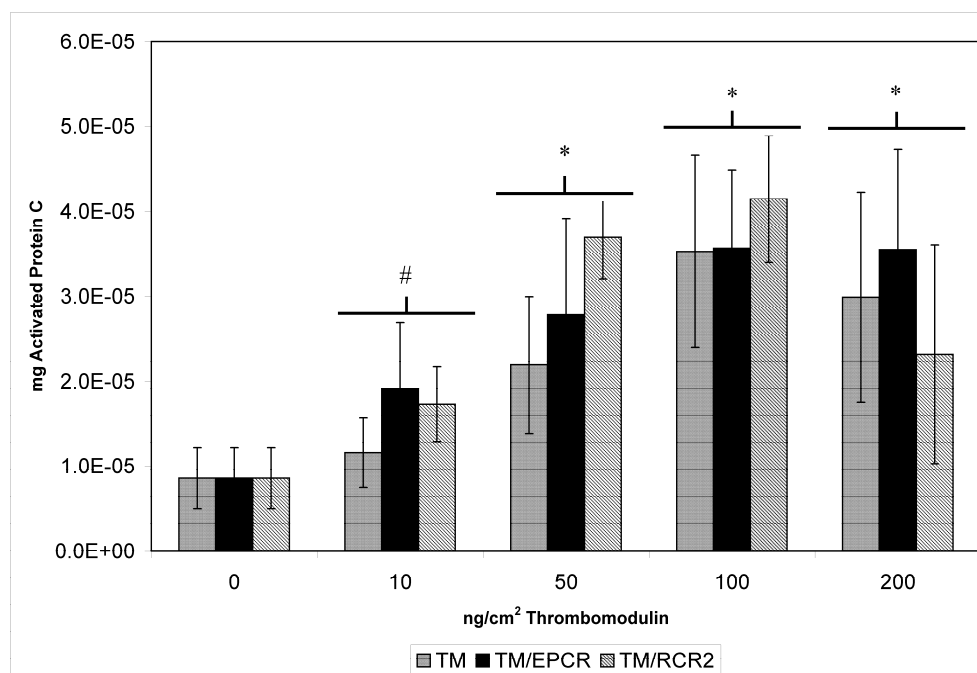


Figure 4: PC activation on UltrabindTM membrane bearing EPCR and TM.

The following protein samples were immobilized on UltrabindTM discs: TM:thrombomodulin alone; rEPCR: rEPCR alone; TM+rEPCR: co-immobilization of TM and EPCR, in a random fashion and (TM+RCR-2) + rEPCR:co-immobilization of TM and RCR-2, in a random fashion, followed by binding of rEPCR to RCR-2. Controls correspond to membranes with no TM or EPCR. EPCR containing samples were spotted in a 7:1 EPCR:TM concentration similar to that seen in vivo. sample-1 (S1): control, no TM or rEPCR; sample-2 (S2): 10 ng/cm² (140 femtomoles) of TM; sample-3 (S3): 50 ng/cm² (700 femtomoles) of TM; sample-4 (S4): 100 ng/cm² (1400 femtomoles) of TM and sample-5 (S5): 200 ng/cm² (2800 femtomoles) of TM. Samples were incubated for 30 minutes at 37 °C with 50 µl of a 5 units / ml thrombin solution. Following incubation,

the thrombin solution was removed and a 50 μ l of a 0.025 mg / ml hPC solution was added to each sample and incubated at 37 oC for 1.5 hours. The reaction was stopped with the addition of 10 μ l of anti-thrombin III to a final concentration of 6 units/ml and incubated for 15 minutes. Subsequently, the solution was pipetted from the solid samples to new 96 well plate. To each solution sample, 40 μ l of Spectrozyme aPC solution was added to a final concentration of 0.004 M and incubated at 37oC and absorbance at 405nm-1 was measured as a function of time. # and * denote statistically different from each other, $p < 0.05$.

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Chapter 4

Development of a Biomimetic Vascular Surface by the Sequential Co-immobilization of Thrombomodulin and EPCR on Polyurethane.

Abstract

In the normal endothelium; thrombomodulin (TM) and endothelial protein C receptor (EPCR), are well-integrated to regulate the protein C (PC) anticoagulant pathway. The central goal of this study was to examine the influence of co-immobilizing rEPCR with TM either randomly or in a sequential fashion, on biomaterial surfaces, on the activation of PC. As TM and EPCR work in concert, co-immobilization on the nanometer scale was desired to anchor the two proteins to enable the desired enzymatic activation when compared to random immobilizations. Polyurethane discs were surface modified to enable the ordered immobilization of proteins via a bidentate moiety, which includes reactive oxirane and carboxylic functional groups. Random immobilizations were carried out via the oxirane arm of the bidentate moiety and all surfaces were prepared with the same defined surface concentrations of rEPCR and TM. Random co-immobilizations of TM and rEPCR on modified PU surfaces yielded increased APC levels when compared to immobilization of TM, however the increases were statistically insignificant. In contrast, sequential co-immobilizations of TM and rEPCR via the bidentate moiety on PU, which allows for immobilization at a molecular proximity, yield APC levels that were significantly ($p < 0.05$) different from immobilizations of TM alone. Further studies were conducted with protein immobilized surfaces and platelet poor plasma using a thromboelastograph to monitor the formation of fibrous clot formation in comparison to control unmodified surfaces and surfaces immobilized with common surface treatments such as albumin and heparin.

Introduction

Polymers such as polyesters, polyurethanes and polytetrafluoroethylene are often used for vascular implants because they are nontoxic with mechanical properties similar to those of vascular tissues.[1, 2] However these polymer implants, like most other synthetic materials, can lead to thrombosis when subjected to blood.[3] In the case of large diameter vascular implants these properties are harnessed to form a polymer fibrous composite graft, however for intermediate and small diameter grafts these thrombi lead to occlusions and failure of the implant.[2] To overcome this phenomena, the surface of the polymer is chemically modified to passivate the surface, often by immobilizing molecules which alter the surface charge or hydrophobicity of the material.[4, 5] Other surface modifications have immobilized anticoagulants such as hirudin[6] or biomolecules such as cholesterol[7]. Because the endothelial membrane of vascular tissue is naturally non-thrombogenic,[8] several studies have been conducted immobilizing membrane proteins and molecules known to inhibit fibrin formation or platelet adhesion including heparin, thrombomodulin, prostaglandin and nitric oxide.[9-15] These coating have shown promise because they are able to interact with the natural molecules used to control haemostasis in the blood such as anti-thrombin III and protein C.[16, 17]

While immobilizing a single protein or biomolecule on to the surface of a biomaterial allows it to mimic one specific pathway, the regulation of haemostasis involves many pathways working in concert.[8] Because of this, studies have attempted to create surfaces which incorporate multiple aspects of the endothelial cell's natural

regulatory molecules. In two of these studies, a fibrin inhibitor such as heparin or thrombomodulin was co-immobilized with a platelet inhibitor such as prostaglandin or a nitric oxide producing molecule.[13, 18] In another study, both thrombomodulin and heparin were immobilized onto surface with a phospholipid membrane.[10] This membrane was meant to mimic the cell membrane inhibiting nonspecific protein binding and also enhance the thrombomodulin function by creating the proper alignment and present the proper environment for the transmembrane region of the protein.

Several of these studies have immobilized thrombomodulin because of its ability to activate Protein C when bound to thrombin.[19] Protein C, once activated, inhibits the formation of Factor Va and Factor VIIIa, which stops the production of thrombin in the common pathway of the clotting cascade.[20] However, naturally the function of thrombomodulin is enhanced by endothelial protein C receptor (EPCR), figure 1.[21, 22] EPCR is a transmembrane protein which has been shown to increase the transport of Protein C onto the material surface and when blocked has shown an 86% decrease in Protein C activation.[23] Crystallographic studies of Protein C bound to EPCR show that when bound Protein C is in presented in an orientation that allows it to be activated by the thrombomodulin-thrombin complex suggesting that Protein C is activated while still bound to EPCR.[24] This mechanism has been further confirmed by incubating Protein C with and without soluble EPCR prior to activation in solution phase, with the result of both having the same reaction rates.[25] Should this mechanism hold true, it would require that the EPCR and thrombomodulin be localized together on the surface. On the cell membrane, Protein C bound to EPCR has been determined through fluorescence energy transfer to be at a height of approximately 94 Å and thrombin bound to

thrombomodulin at approximately 64 Å above the cell membrane.[26] If these protein complexes were approximated as a sphere with the height as the diameter where the reaction sites are able to move along the surface of the sphere this would require that EPCR and thrombomodulin be immobilized on a surface by a separation of less than 80 Å, or 8 nm.

Different techniques have been developed to pattern immobilize biological molecules onto the surface of materials. Techniques such as photomask lithography, nanografting and nanoimprinting have all been used to create patterns onto polymer supports, however these techniques all have limitations. Photomask lithography, a technique where a monolayer or polymer is coated either physically or chemically with photo-active polymer and a light source through a patterned cover immobilizes the target protein in the pattern on the surface.[27-29] This method is limited by low resolution on the mask and has been limited to micron and submicron patterns. Nanografting is a technique where proteins are loaded onto an AFM probe and then touched to the surface in the desired pattern.[30] While this method does offer the resolution necessary, it cannot be easily scaled to a manufacturing level. Nanoimprinting is a technique where a stamp is created through one of the other lithography techniques and is printed onto the surface through direct contact.[31] The limitations of this technique are the life of the stamp, the resolution of the protein pattern during the stamping process and the alignment needed for the stamp or multiple stamps in the case of co-immobilization.

This research proposes the co-immobilization of thrombomodulin and EPCR onto a polyurethane support. In this method, figure 2, the two proteins will be immobilized using a bidentate functional group synthesized on the surface of a post

fabricated polyurethane sample across the solid / liquid interface. We hypothesize that by immobilizing thrombomodulin and EPCR onto the surface via the bidentate functional group, that the distance between the two proteins can be controlled such that the activity of thrombomodulin on the surface is enhanced as measured by Protein C activation in comparison to blank surface, surfaces modified with thrombomodulin alone and samples with a random co-immobilization of the two proteins. We further hypothesize that the co-immobilization of thrombomodulin and EPCR will delay the formation of fibrous clots.

Methods

Chronoflex AR medical grade polyurethane was generously provided as a gift from Cardiotech Inc. (Cambridge, MA).

2.1 Preparation of Surface Bridge

The chemical representation of the proposed surface modification scheme is shown in figure 2. Reaction 1 will enable the addition of the first reactive oxirane moiety to the secondary nitrogen incorporated in the carbamate subunit of the PU backbone. The addition of the reactive carboxylic acid branch of the bidentate bridge is achieved in reaction 2. Reaction 3 enables the addition of a reactive oxirane functional group to complete the bidentate bridge. Subsequent paragraphs will provide the experiment details of the surface modification.

2.1.1 Reaction 1

Solution cast polyurethane films were cut into 0.5 cm diameter samples with an average weight of 30 mg using a standard biopsy punch. Under an inert atmosphere, approximately 1 gram of polyurethane was added to 75 mL tetrahydrofuran (THF, HPLC Grade 99.9%, Sigma Aldrich, St. Louis, MO) mixed with 75 mL hexanes (ACS Grade, Sigma Aldrich, St. Louis, MO) and the temperature of the mixture reduced to -5 °C using an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized

with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.2 Reaction 2

PU samples from reaction 1 were added to a 0.02 M 5-amino valeric acid (Sigma Aldrich, St. Louis, MO) in water. The samples were allowed to react at room temperature for 24 hours with stirring. Samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times. All absorbed water was removed by evaporation under reduced pressure, $P < 0.3$ Torr for at least 6 hours.

2.1.3 Reaction 3

Under an inert atmosphere, PU samples from reaction 2 were added to a 150 ml mixture THF and hexanes (50/50 by volume) and the temperature of the mixture reduced to -5 °C in an isopropyl alcohol ice bath. 0.5 mL epibromohydrin and 0.5 mL of a 2.0 M lithium diisopropyl amine (LDA, Sigma Aldrich, St. Louis, MO) in THF were added to the mixture and the reaction was allowed to proceed for 2.5 hours with stirring. Upon completion, the LDA, THF solution was removed from the PU samples and neutralized with a 3% (V/V) acetic acid solution. The PU samples were washed with 150 mL deionized water for 30 minutes and repeated 3 times to remove excess THF / Hexane solution. Residual solvent was removed under reduced pressure, $P < 0.3$ Torr for at least 6 hours. Samples were at room temperature until use.

2.2 Protein Immobilization

Protein solutions of recombinant EPCR-his, human thrombomodulin (TM, American Diagnostica Inc., Stamford, CT) and rabbit lung thrombomodulin (rTM, American Diagnostica Inc, Stamford, CT) were prepared in PBS buffer (pH 7.2).

2.2.1 Protein 1 via Carboxylic acid

A 0.1 g / mL solution of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, Thermo Fisher Scientific, Rockford, IL) was prepared in a 0.1 M 2-(N-Morpholino)ethanesulfonic acid (MES, Sigma Aldrich, St. Louis, MO) solution pH 4.5. 500 μ L of the EDC in MES solution was added to each sample and was allowed to react at room temperature with shaking. After 15 minutes, 500 μ L of a 20 mg / mL *N*-hydroxysulfosuccinimide (Sulfo-NHS, Thermo Fisher Scientific, Rockford, IL) in MES buffer and added to each sample's solution. After 30 minutes at room temperature, the solution was removed from each sample and 100 μ L of protein 1 in solutions prepared in phosphate buffered saline (PBS, pH7.2) was added to each PU samples from reaction 3 for 6 hours at room temperature. Upon completion, the PU samples were washed with 2 mL of PBS per sample 2 x 30 minutes.

2.2.2 Protein 2 via Oxirane

Following the immobilization of protein 1, 100 μ L of protein 2 prepared in PBS was reacted for 24 hours at 4°C. The samples were washed 2 x 30 min PBS, 2 x 30 minutes PBS +.05% Tween 20, 3 x 30 min PBS.

Following final protein attachment the samples were stored in reaction buffer composed of 0.1 M NaCl (Sigma Aldrich, St. Louis, MO), 0.003 M CaCl₂ (Sigma Aldrich, St. Louis, MO), 0.0006 M MgCl₂ (Sigma Aldrich, St. Louis, MO), 0.02 M Tris-HCl (Fisher Scientific, Pittsburgh, PA), 0.1 mg/ mL Bovine Serum Albumin (BSA, Sigma Aldrich, St. Louis, MO) and the pH adjusted to 7.5. at 4°C.

2.3 Solid Phase Protein C Activation Assays

2.3.1 Solid Phase Protein C Activation Assays Low Thrombin Concentration

Samples prepared previously were washed in 50 µl fresh reaction buffer prior to reaction. Samples were incubated for 30 minutes at 37 °C with 50 µl of a 5 units / ml Thr solution with mixing. Following incubation, the thrombin solution was removed but the samples not washed. 50 µl of a 0.025 mg / ml hPC solution was added to each sample and reacted at 37 °C with mixing for 1.5 hours. The reaction was ended by the addition of 10 µl of anti-thrombin III (American Diagnostica Inc., Stamford, CT) to a final concentration of 6 units / ml. Following 15 minutes of reaction of anti-thrombin with thrombin from the reaction, the solution was pipetted from the solid samples to new 96 well plate. To each solution sample, 40 µl of Spectrozyme aPC solution was added to a final concentration of 0.004 M. Solution samples were incubated at 37°C analyzed 405 nm⁻¹.

2.3.2 Solid Phase Protein C Activation Assays High Thrombin Concentration

Samples prepared previously were washed in 50 µl fresh reaction buffer prior to reaction. Samples were incubated for 30 minutes at 37 °C with 50 µl of a 5 units / ml Thr

solution with mixing. Following incubation, 50 μl of a 0.025 mg / ml hPC solution was added to each sample and reacted at 37 °C with mixing for 1.5 hours. To end the reaction, the solution was pipetted from the solid samples to new 96 well plate. To each solution sample, 25 μl of Spectrozyme aPC solution was added to a final concentration of 0.004 M. Solution samples were incubated at 37°C and analyzed at 405 nm.

2.4 Thromboelastograph

Platelet poor plasma (PPP) was prepared from fresh, drawn less than 36 hours prior to usage and never frozen, whole citrated blood (Research Blood Components LLC, Brighton, MA) prior to each experiment by centrifuging whole citrated blood at a force of 2300 x g for 15 minutes at room temperature. Samples were inserted into disposable chambers which had been bored out to a diameter of 0.406 inches and 425 μL PPP was added. Clotting was initiated with the addition of a 200 mM CaCl_2 60 mM MgCl_2 solution to a final calcium concentration of 6.0 mM. Samples were analyzed using a Thromboelastograph Analyzer 5000 (Haemoscope, Niles, IL) for speed of onset of the clot, initial formation rate of the clot and maximum strength of the formed clot.

Results

3.1 Polyurethane Sample Preparation

The characterization of the bidentate functional group modification to polyurethane samples has been discussed earlier as well as the ability to bind proteins to a specific end of the functional group.

Solid samples were prepared from TM solutions to produce a surface density of TM of 50 ng/cm^2 based upon 50 % completion and considering the samples to be 2 dimensional (no width) and two faces. This value is approximately 4.5 times the surface density found on native vascular endothelial surfaces.[32] The EPCR was added at a 7:1 molar ratio to TM based upon the ratio of the two proteins seen in native tissue.[32] This ratio was kept even in sequential reactions despite the bidentate functional group having a only two reaction sites per functional group. All samples were blocked with ethanol amine and stored under buffer containing BSA to ensure the opening of all oxirane attachment sites.

Because the different chemistries have the potential to bind to different sites on the protein, it was necessary to compare the activity of TM when bound to either the oxirane or acid end of the functional group. As can be seen in figure 3, no significant difference can be seen in the activity of TM. With no significant difference seen in the activity, all samples were prepared with proteins added through the oxirane functional group except in the case of samples with ordered co-immobilization which were prepared with TM immobilized through the acid group and EPCR added through the oxirane.

3.2 Protein C Activation Assay

The protein C activation assay was conducted in two different manners, in the presence of a low concentration of thrombin and in the presence of a high concentration of thrombin. In each of these experiments the initial concentration of thrombin added was the same, however in the low thrombin concentration experiments the thrombin solution was removed after incubation. In low thrombin experiments sample modified with TM and EPCR both through the oxirane were not able to activate Protein C at a rate greater than that of TM alone, similar to what was seen on ultrabind surfaces in the previous chapter. However, samples with the sequential immobilization showed a increase in the activation of Protein C over both the TM alone ($p = 0.05$) and the random co-immobilization to TM and EPCR ($p < 0.001$). In high thrombin experiments, both the sequentially ordered ($p < 0.001$) and the randomly immobilized ($p = 0.02$) showed a significant increase in the ability of the surface to activate Protein C, however in this experiment as well the sequentially ordered samples were able to activate significantly more Protein C than randomly immobilized samples ($p < 0.05$).

3.3 Thromboelastograph

Thromboelastography (TEG) is a method of testing the efficiency of coagulation in the blood and measures the changes in elasticity at all stages of the developing and resolving clot. To evaluate the effect of the protein immobilized PU surfaces on the efficacy of coagulation, samples were studied by thromboelastography using platelet poor plasma (PPP). The time taken to initial fibrin formation and clot onset were evaluated for modified PU films immobilized with rabbit TM, rabbit TM and EPCR in a random

bonding and rabbit TM and EPCR in a sequentially ordered immobilization. These samples were compared to modified films and films immobilized with BSA or Heparin, two standard non-thrombogenic coatings. The data was plotted in figure 6 and the data for initial fibrin formation (SP) and time to clot initiation (R) plotted in figures 7 and 8 respectively with the values normalized to control bare surfaces show in figure 9.

Because of restraints on both the polymer material and the amount of recombinant protein available and because the samples could not be reused following the exposure of PPP prepared from human blood, this experiment was run only twice with samples in triplicate. Because of the small number of data points and the experiment being run only twice, it was difficult to establish high confidence limits for this experiment. As a result data was compared as established for $p < 0.03$, suggested $p < 0.10$ and possible for $p < 0.20$. These relationships for TM, TM / EPCR random and TM / EPCR ordered compared the controls and each other are described in figure 10.

Discussion

There is a significant need to design vascular grafts which contain similar mechanical properties of natural vascular tissue and are non-thrombogenic when contacted with blood. While different biomedical polymers have been used to approximate the mechanical properties of native arteries, these polymers have been shown to be thrombogenic, requiring surface modifications to pacify the surface to blood contact. As a result of this need, several groups have studied the native endothelium of vascular tissue and attempted to mimic its methods of controlling haemostasis. In these studies, endothelial membrane bound molecules such as heparin or thrombomodulin (TM) have been immobilized onto biomaterials surfaces in the hope of controlling thrombin formation on their surface.[33, 34] However the regulation of haemostasis is complex several surface groups, many of which have cofactors. On large arteries, TM is co-localized with endothelial protein C receptor (EPCR) which is thought to increase the transport of Protein C onto the endothelial surface.[35]

To take advantage of the increased transport of Protein C by EPCR, it was necessary to immobilize EPCR and TM to the bidentate functional group to maintain a physical distance which could be bridged and allow the reaction to proceed. Our initial tests were to determine if the activity of TM changed when bound to each of the reactive sites of the bidentate functional group, figure 3. With no significant difference, the ability of TM to activate Protein C was studied on surfaces with TM alone, TM and EPCR in a random orientation and TM and EPCR immobilized sequentially to one side of the functional group. The static tests were conducted in two separate manners based upon the total thrombin concentration. Previous studies have incubated their samples

with thrombin to allow the binding of thrombin to TM on the surface and then removed the thrombin solution prior to Protein C activation. This could be to avoid the possibility of Protein C activation by unbound thrombin which is a very slow reaction. However the binding of thrombin to TM is a reversible process and with the removal of the thrombin solution there is no boundary concentration of free thrombin. Because of the lack of a boundary layer concentration close to the material surface, and thrombin that is released during the reaction will not be reabsorbed onto the surface. This phenomena does not follow the process in vivo where thrombin production is on the injured cell or biomaterial surface. To better model this event, the excess thrombin solution was included in the reaction mixture during Protein C activation. As can be seen in figure 4, a significant increase was seen in Protein C activation in both low and high thrombin experiments for samples with ordered immobilization over samples immobilized with TM alone and TM and EPCR in a random orientation. While the increase was significant, it was not the 7x increase that is seen on arteries.[35] This could be do to several factors, first the alignment of EPCR and TM was not controlled, so while the distance was controlled the proteins could be could be bound to the surface in a manner that blocks the reactive site. Another possible reason for the small increase is that despite the 7:1 immobilization of EPCR to TM on the surface which is based upon the concentration of the two proteins on large arteries[32], the bidentate functional group is only 1:1 meaning that largest concentration of EPCR is most likely not immobilized at a distance which can directly interact with TM.

Both of these concerns should have been addressed by the immobilization of RCR2, an antibody against EPCR, with TM in an ordered configuration. RCR2 binds

EPCR on the cytosolic portion of the protein near the transmembrane region meaning that bound EPCR should be in the proper orientation. RCR2 also has the ability to bind EPCR to both Fab regions of the antibody meaning it could bind 2 molecules of EPCR for every RCR2 molecule. However samples with RCR2 showed a significant decrease when compared to samples with TM and EPCR alone, figure 5. This decrease in activity was believed to be the result of steric interference from too high a density of proteins for the thrombin and Protein C to bind and migrate to an alignment to react.

Polyurethane samples with immobilized proteins were analyzed for their affect on fibrin clot formation by thromboelastograph (TEG) using platelet poor plasma (PPP). PPP was used to eliminate the effect of platelets on thrombus formation to prove whether co-immobilized EPCR with TM would have an effect on fibrin formation. Two parameters were focused on, SP which is the time when the TEG first detects thrombin formation and R which is the reaction time measured at the time of first significant clot formation. In both of these measurements, times were significantly increased for samples with EPCR co-immobilized with TM over bare polymer or polymer immobilized with bovine serum albumin, a method of pacification, figures 7-10. It is difficult to compare EPCR /TM samples to TM alone as this test was only able to be run twice with samples in triplicate due to restrictions on the quantity of both polymer and recombinant proteins and TM samples while had agreement to their test group, created a large standard deviation upon the two sets being unified. While no significant difference could be detected between EPCR / TM in a random immobilization and samples with either TM alone or with the same number of pharmaceutical units of immobilized heparin. A possible difference could be seen when comparing TM / EPCR in an ordered

immobilization to samples of TM alone and samples with heparin, however more testing would be necessary to confirm these results.

Also of interest is the ratio of increase over control samples for TEG samples as compared to the Protein C activation assays. For the SP measurement the ratio of Ordered compared to TM alone was 5.7:1 and the for the R values 2:1 where as for the activation assays ordered samples averaged 1.5:1. While these values do measure different processes, these processes are related as they both are products of the regulation of thrombin. Since the thrombin concentrations on the surfaces of the samples are the same, the change must be related to an increase in the EPCR transport.

Conclusions

With the growing development of biomimetic surfaces, the co-immobilization of proteins and their cofactors onto surfaces need to be studied to better interact with the body and its physiology. To date, these methods have been limited to random immobilizations which do not ensure that the immobilized molecules are able to react with each other as they would in the body.

We have demonstrated a method to immobilize thrombomodulin and endothelial Protein C receptor using a bidentate functional group extended from the surface of polyurethane in an effort to create a less thrombogenic surface. Samples immobilized with this protein system have demonstrated a greater ability to activate Protein C in both high and low thrombin systems. Finally, we have shown that the ordered system is able to delay fibrin formation and fibrous clot formation over unmodified polymer, polymer with an immobilized BSA coating and samples immobilized with thrombomodulin alone. We have also show that samples with this system inhibits clot formation as well as and possibly better than a polyurethane with a heparin coating.

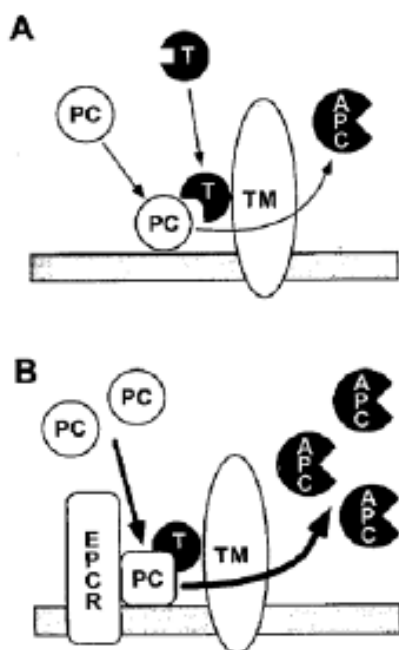


Figure 1 Representation of Protein C Activation on the Vascular Endothelium

Representation of the mechanism of activation of the anticoagulant Protein C by a) thrombomodulin (TM) alone and b) thrombomodulin and endothelial protein C receptor (EPCR)

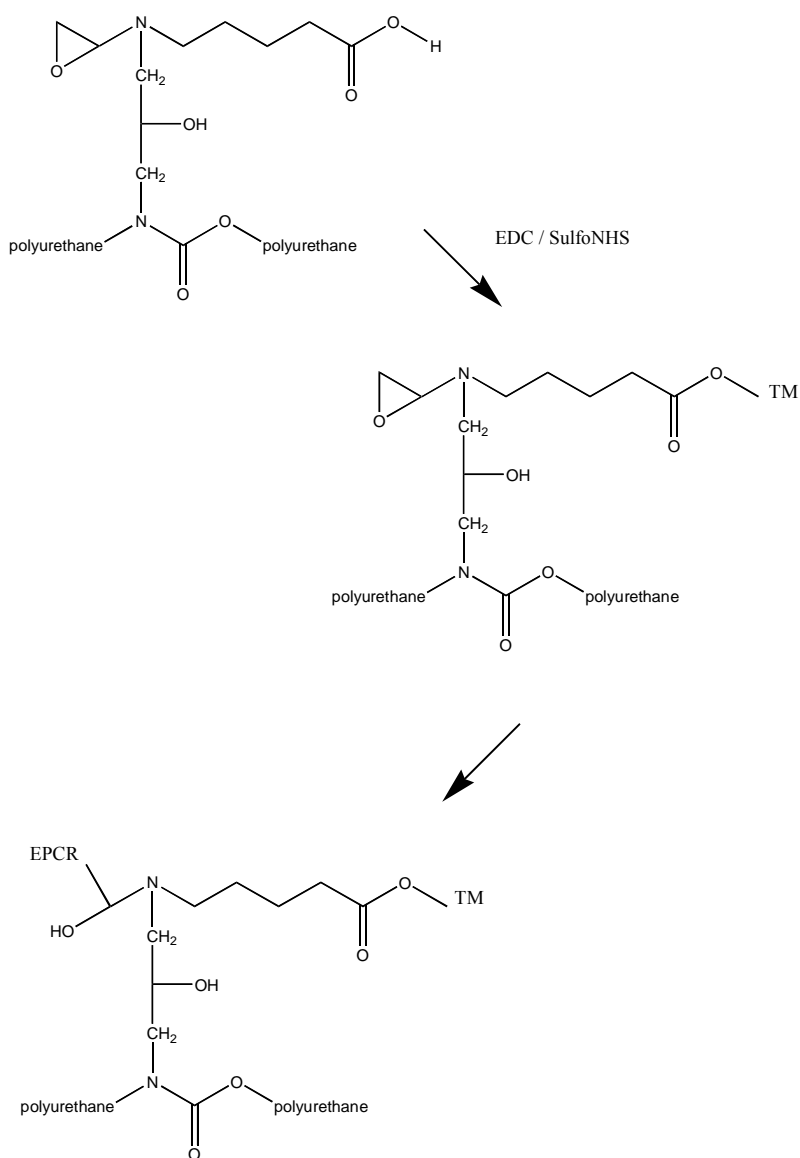


Figure 2 Chemical Representation of Thrombomodulin and EPCR to Polyurethane through the bidentate functional group.

Covalent immobilization of the proteins TM and EPCR via the bidentate functional group for ordered immobilization. This representation demonstrates TM being immobilized through the carboxylic acid group and EPCR through the oxirane as was the case in the data reported in this chapter, however immobilization of EPCR through the acid and TM through the oxirane yielded no statistical difference (data not shown).

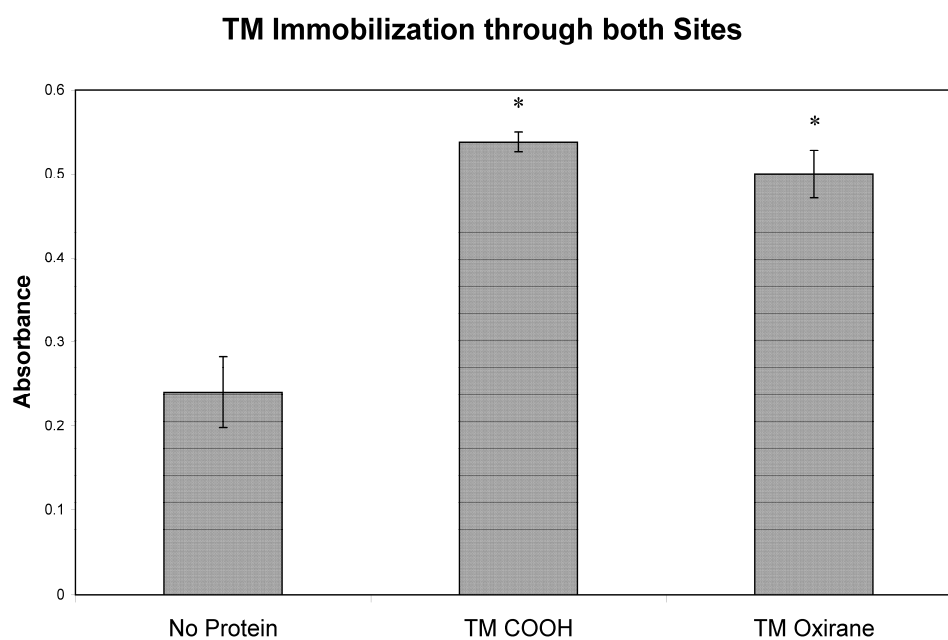


Figure 3: Functional activity of TM immobilized on PU via reaction 2 and reaction 3.

In separate experiments, a desired concentration of 100 ng/cm² (1400 femtomoles) of TM was immobilized via –COOH group (reaction 2) and via the oxirane group (reaction 3). Functional activity of immobilized TM (i.e PC activation in the presence of thrombin) was assayed by incubating samples with thrombin for 30 minutes and procedure as detailed in legend to figure 4 was followed.

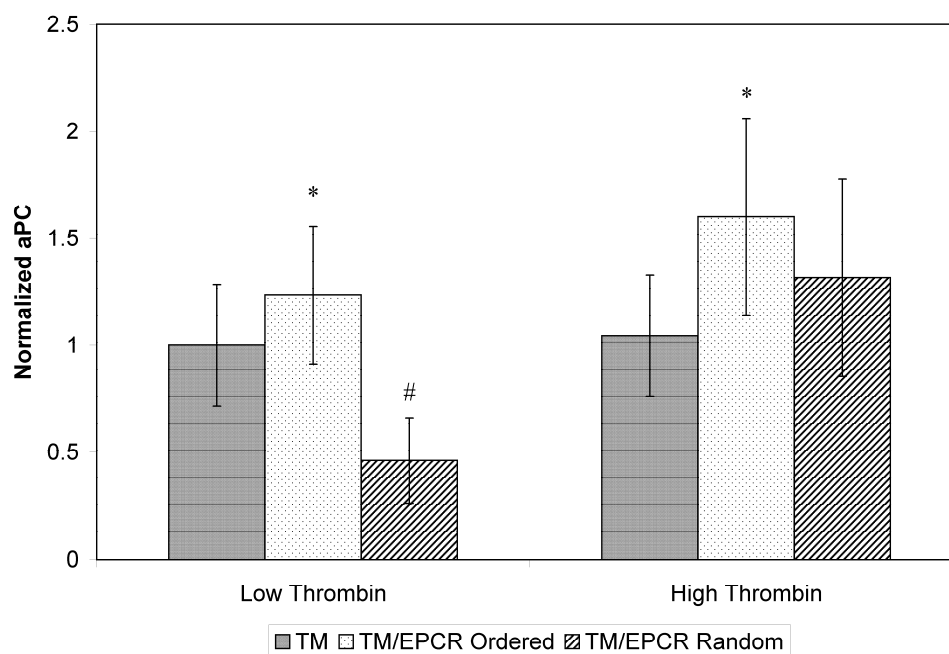


Figure 4 Protein C Activation

Protein C activation in high and low thrombin concentration experiments by polyurethane samples immobilized with thrombomodulin (TM), thrombomodulin and EPCR randomly immobilized and thrombomodulin and EPCR sequentially immobilized. All values normalized to concentration of activated Protein C (aPC) produced by polyurethane samples immobilized with thrombomodulin alone. * represents statistically higher than control ($p < 0.05$)

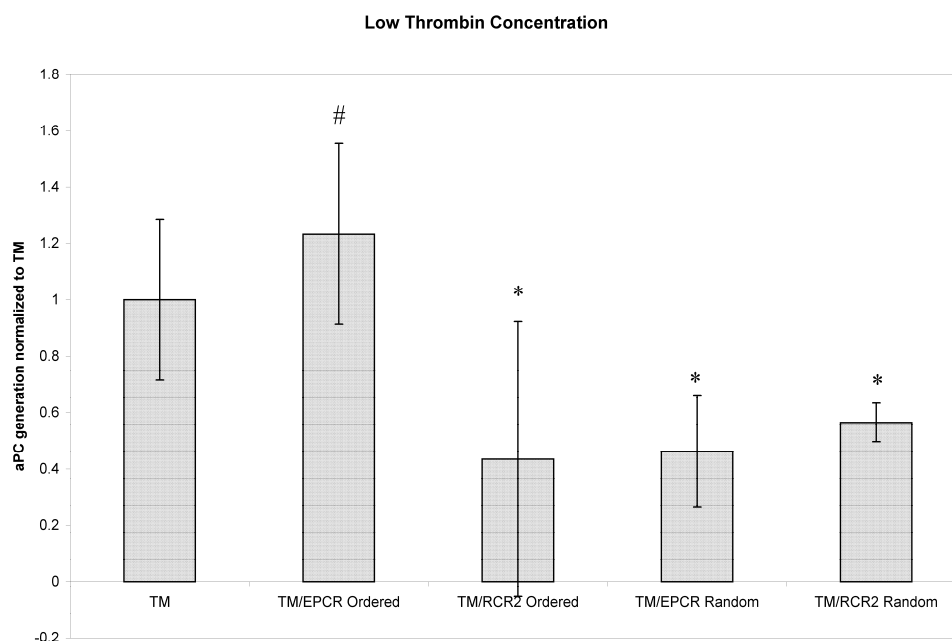


Figure 5: PC activation on PU surfaces bearing EPCR and TM.

The following protein samples were immobilized on PU discs: TM:thrombomodulin alone; TM+rEPCR: co-immobilization of TM and EPCR, in a random fashion; TM/rEPCR: sequential immobilization of TM and EPCR, in a directed fashion; (TM+RCR-2)+rEPCR: co-immobilization of TM and RCR-2, in a random fashion, followed by binding of rEPCR to RCR-2 and (TM/RCR-2)+rEPCR: sequential immobilization of TM and RCR-2, in a directed fashion, followed by binding of rEPCR to RCR-2. Controls correspond to membranes with no TM or EPCR. EPCR containing samples were spotted in a 7:1 EPCR:TM concentration similar to that seen in vivo. A 2:1 rEPCR:RCR-2 stoichiometry and 20% antigen binding efficiency was assumed in calculating the amount of RCR-2 immobilized (750 ng/cm²). Functional activity of surfaces bearing TM and EPCR (i.e PC activation in the presence of thrombin) were assayed using the low thrombin method. # and * denote statistically different from each other, $p < 0.05$.

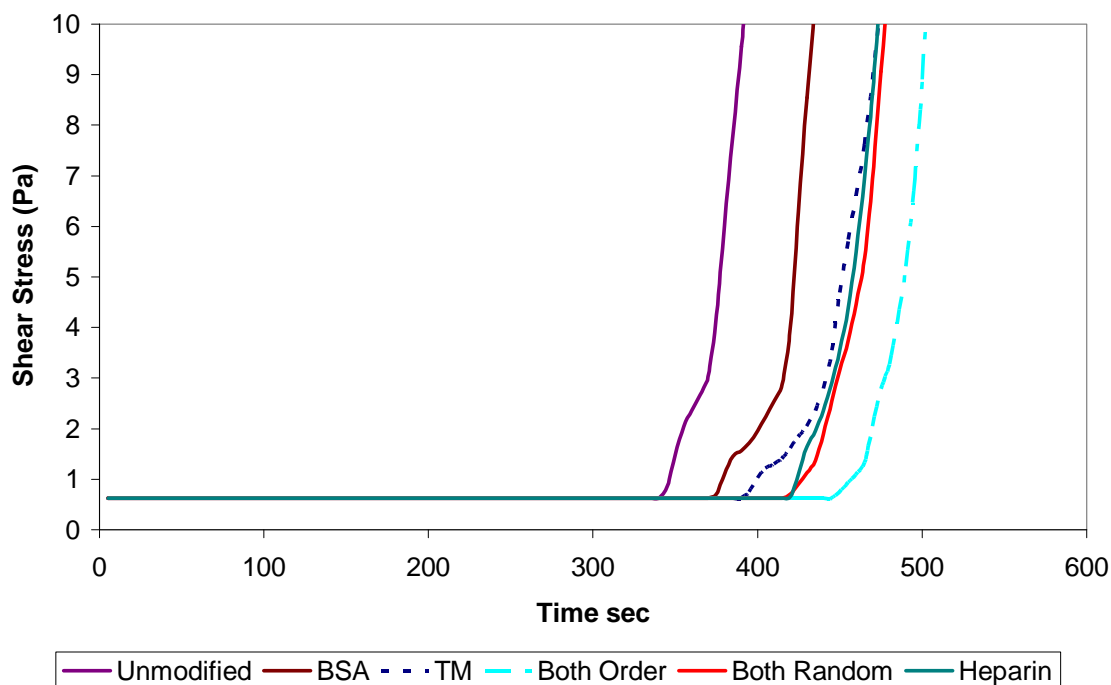


Figure 6: Thromboelastograph Representative Data

Modified PU samples immobilized with BSA, TM, TM/EPCR ordered immobilization, TM/EPCR random immobilization and heparin were inserted into disposable chambers which had been bored out to a diameter of 0.406 inches, and 425 μL of PPP was added. Clotting was initiated with the addition of a 200 mM CaCl_2 and 60 mM MgCl_2 solution to a final calcium concentration of 6.0 mM. Samples were analyzed using a Thromboelastograph Analyzer 5000 for time to initial fibrin formation (SP) and time to initial clot formation (R).

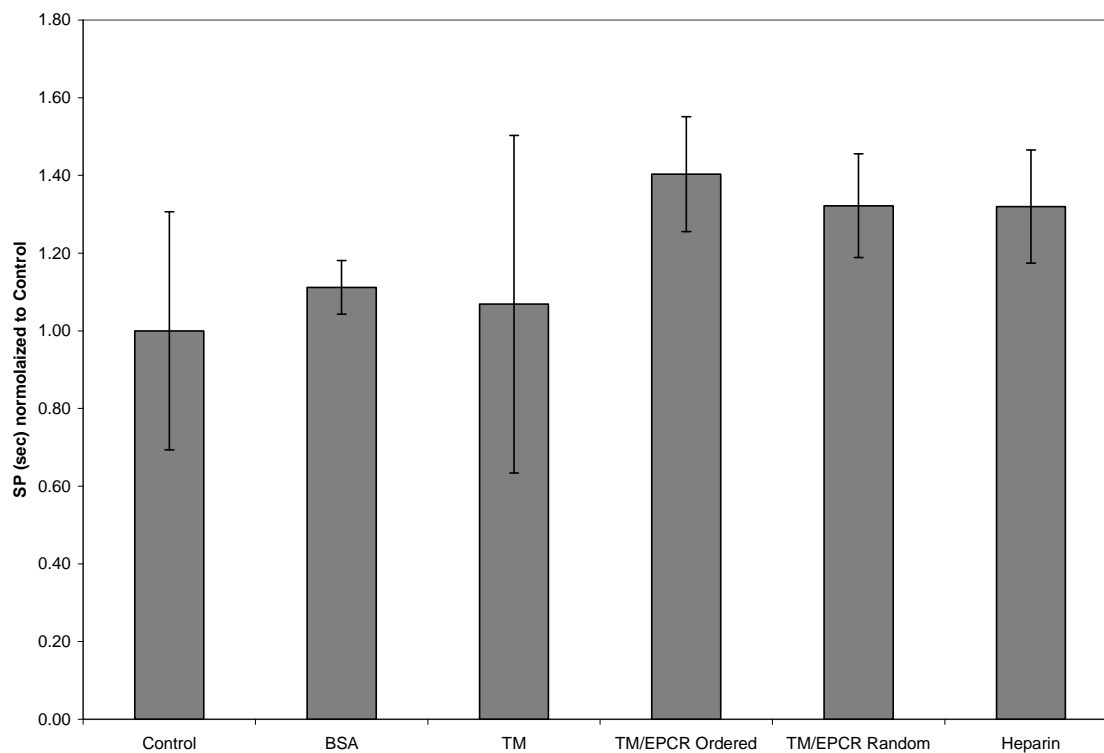


Figure 7: Initial Fibrin Formation

Modified PU samples immobilized with BSA, TM, TM/EPCR ordered immobilization, TM/EPCR random immobilization and heparin were inserted into disposable chambers which had been bored out to a diameter of 0.406 inches, and 425 μL of PPP was added. Clotting was initiated with the addition of a 200 mM CaCl_2 and 60 mM MgCl_2 solution to a final calcium concentration of 6.0 mM. Data reported for time to initial fibrin formation (SP).

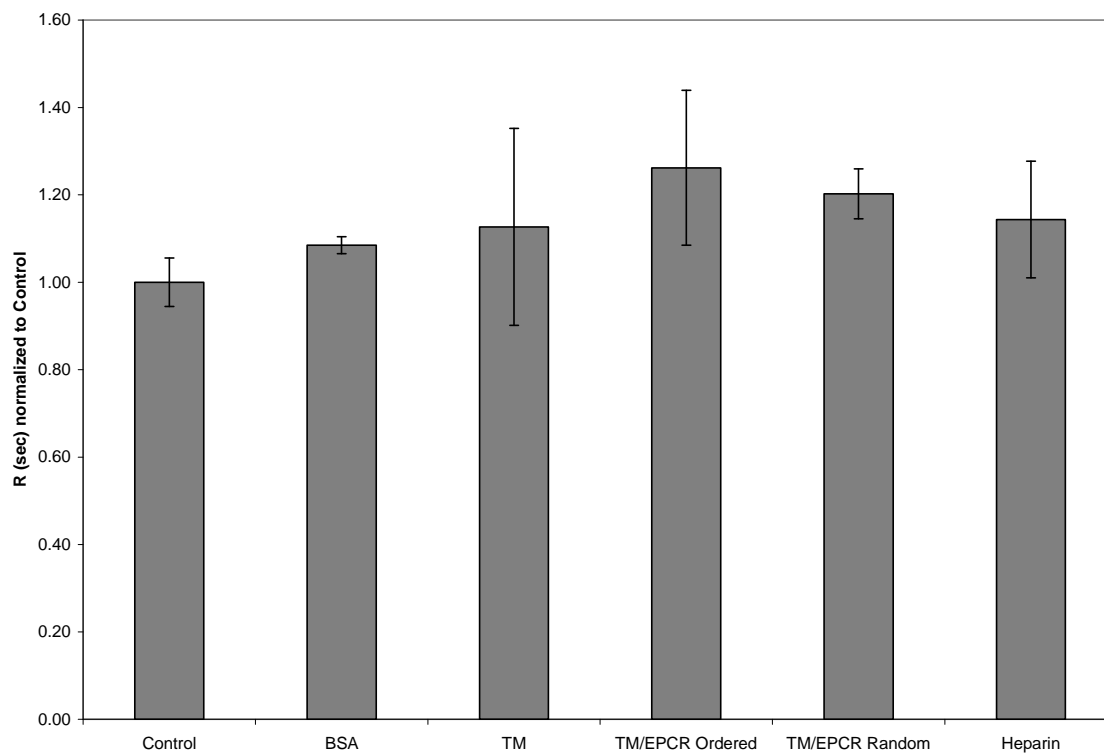


Figure 8 Time to Initial Clot Formation

Modified PU samples immobilized with BSA, TM, TM/EPCR ordered immobilization, TM/EPCR random immobilization and heparin were inserted into disposable chambers which had been bored out to a diameter of 0.406 inches, and 425 μL of PPP was added. Clotting was initiated with the addition of a 200 mM CaCl_2 and 60 mM MgCl_2 solution to a final calcium concentration of 6.0 mM. Data reported for time to initial clot formation (R) which corresponds to the endpoint of an activated partial thromboplastin time (APTT).

	SP (sec)	Std Dev	R (sec)	Std Dev
Control	1.00	0.31	1.00	0.06
BSA	1.11	0.07	1.08	0.02
TM	1.07	0.43	1.13	0.23
TM/EPCR Ordered	1.40	0.15	1.26	0.18
TM/EPCR Random	1.32	0.13	1.20	0.06
Heparin	1.32	0.15	1.14	0.13
Control (Actual)	285.00		346.67	

Figure 9: Values for SP and R

Comparison of values for time to initial fibrin formation (SP) and initial clot formation (R) measured using PPP in contact with PU with different surface immobilizations. Two independent experiments with samples in triplicate were analyzed. Clotting was initiated with the addition of a 200 mM CaCl₂ and 60 mM MgCl₂ solution to a final calcium concentration of 6.0 mM.

		Controls			Samples		
		No Protein	BSA	Heparin	TM	TM / EPCR random	TM / EPCR ordered
TM	SP	N/D	N/D	Possible worse	-	Possible	Suggested
	R	Possible	N/D	N/D	-	N/D	Possible
TM / EPCR random	SP	Established	Established	N/D	Possible	-	Possible
	R	Established	Established	N/D	N/D	-	N/D
TM / EPCR ordered	SP	Established	Established	Possible	Suggested	Possible	-
	R	Established	Established	Possible	Possible	N/D	-

Figure 10: Comparison of clotting parameters (SP and R).

Due to limited number of samples for thromboelastography experiments, 2 runs with samples in triplicate, the following constraints were made for analyzing potential differences between sample types. $P < 0.03$ denotes that statistical difference between sample and control was established. $P < 0.1$ denotes a statistical difference between sample and control was possible. $P < 0.2$ denotes a statistical difference between sample and control was suggested. N/D not determined.

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Future Experiments Suggested for the Thrombomodulin-Endothelial Cell Protein C
Receptor Functionalized Surface and a Sequentially Immobilized Surface

Future Work

This dissertation has presented an approach for the development of a novel biomimetic vascular surface, however there are several areas which must be studied before this could be developed into a potential vascular graft. This chapter will describe possible future directions for the both the EPCR / TM endothelial cell mimicking surface as well as the other possible projects for the sequential immobilization chemistry.

Experiment Set 1: Determine the effects of flow on the production by the functionalized surface of the anticoagulant Activated Protein C. I predict that more Protein C will be activated in tubing functionalized with the EPCR / TM complex than in native tubing or tubing functionalized with TM or EPCR alone. I also predict based upon the conclusions formed in chapter 4, that the patterned immobilization will show more activity than a random co-immobilization, especially in low concentrations of thrombin.

In these studies commercially available polyurethane (PU) tubing constructed from Chronoflex AR will be purchased from Cardiotech Inc. Following the procedures developed in chapter 1, the luminal side will be functionalized and immobilized with both TM and EPCR, TM alone and BSA. PC reaction buffer described in chapter 3 and 4 with spectrozyme added and a standard UV chromatography sensor analyzing at 405 nm will be used to determine the concentration of PC activated. Activation will be analyzed on surfaces which are pre-incubated for 1 min with thrombin solution (low thrombin experiment chapter 3 and 4) and with thrombin incorporated (high thrombin experiment) as well as at various flow rates in the arterial and venous flow regimes. Two control

surfaces will be incorporated into the experiment, unmodified PU and BSA immobilized PU. The BSA immobilized will serve two separate purposes as a control, first BSA coated surfaces are in general considered to non-thrombogenic. The second purpose of the BSA immobilized sample is that it will act as a control of protein adsorption onto the bare PU surface creating improper measurement.

Experiment Set 2: Determine the immunogenicity of recombinant EPCR produced in *Pichia pastoris*. While recombinant proteins produced in *Pichia pastoris* have glycosylation patterns at the same positions as mammalian proteins and shorter than bacteria and other yeast, the glycosylation pattern is still recognized by the human immune system as derived from fungi and as such can initiate an immune response. It is necessary to determine how the immune system will react to recombinant EPCR as an adverse immune response can lead severely decrease life expectancy of the proteins on the surface or rejection of the implant. I predict that recombinant EPCR grown in *Pichia* will lead to the activation of the immune system.

In this experiment, recombinant EPCR in both glycosylated and deglycosylated form will be incubated in separate samples over mouse macrophages cultured on a tissue culture plate. Following incubation the macrophages will be examined for activation. In another experiment, solution suspended macrophages will be incubated with surface bound EPCR. Should both the glycosylated and deglycosylated forms of recombinant EPCR activate mouse macrophage, clones for recombinant EPCR produced in Chinese Hamster Ovary (CHO) or humanized *Pichia* cells. The products of these cells will then be tested for both immune response and activity.

Experiment Set 3: Determine the long term stability of the protein complex formed and immobilized on functionalized surface. The longterm stability of any surface coating must be determined to understand the life expectancy on an implantable device. Previous studies have shown that TM immobilized surface show a loss of activity which stabilizes after 3 months.[1] Studies of PU immobilized samples showed that sequentially immobilized samples degraded at a slower rate than did TM alone immobilized samples though not enough samples or readings were included to create a statistically relevant experiment and as such this data was not included within this dissertation. I predict that as was seen in previous samples that sequentially immobilized samples will degrade at a slower rate and that the ration of aPC produced will increase in comparison to samples with TM immobilized alone.

This experiment will include PU immobilized with TM alone, TM and EPCR and TM and a completely deglycosylated EPCR. Samples will be designed for both static and flow experiments and analyzed at two week intervals to determine PC activation. When not being used for experiments, samples will be kept at 37°C and stored in Hank's Buffered Saline solution with 1% BSA. Samples will be incubated at 37°C to simulate implanted conditions. A second experiment should include incubation with platelet poor plasma (PPP) to test for stability against proteolytic damage to the immobilized recombinant proteins. In this experiment citrated blood should be used to prevent fibrin formation on the surface which could create an artificial fouling of the surface. Prior to each experiment samples should be washed for 24 hours with recirculating sterile PBS with 1% BSA to wash samples followed by experimentation to determine PC activation.

Experiment Set 4: Characterize the blood compatibility of the functionalized surface including platelet adsorption and thrombus formation in situ. We predict that platelet adsorption will be similar on all functionalized surfaces and that the EPCR / TM functionalized surface will have a delayed clotting time, similar to that seen in our preliminary results. In addition it is believe that any thrombi formed upon reaction with EPCR/TM immobilized samples will have a less stable clot formed when compared to control samples, those immobilized with TM alone or BSA.

Experiments will be conducted first with washed platelets, then with platelet rich plasma (PRP). In both static and flowing experiments, samples will be tested for platelet adhesion and activation. In addition this experiment will thrombin production levels following incubation with platelets. In this method, the ability surface activated PC to arrest thrombin formation will be studied with platelets which can once activated form a surface for thrombin activation.[2, 3] In other studies, thromboelastograph (TEG) studies will be conducted on protein immobilized surface with PRP and with whole blood. Studies with whole blood are important as in coagulation in vivo, it is often found that red blood cells become entrapped in the fibrous clot creating a more stable clot. It is believed that clots on TM/EPCR surface will be less stable because the greater quantities of PC will be activated limiting thrombin formation formation and fibrin formation and crosslinking.[4]

Experiment Set 5: Evaluate the function of the protein immobilized surfaces in vivo.

Because of the nature of drawn blood to require the addition of an anticoagulant, it is necessary for flow experiments to be conducted in vivo. I predict that the activation of

Protein C will occur in vivo similarly to those experiments in situ which will delay or prevent thrombus formation.

In situ experiments can only give so much information on the actual enzymatic responses of blood and the process of haemostasis. Protein functionalized tubing will be implanted into excised portions of the femoral artery and large diameter vein of a rat model. Samples will be impanted for different intervals between 1 hour and 24 hours. Following experiments rats will be sacrificed and the implants excised for analysis. Following sufficient washing samples will be analyzed to activated platelets, fibrous clot formation and occlusion.

Experiment Set 6: Utilize the sequentially immobilized surface to create a cell seeded and directed vascular surface. The seeding of cells onto a surface requires a binding point on the material surface. This binding point can include a physical depression in the material surface, a surface bound peptide or absorbed proteins cover the surface. While cells will bind to the surface, most cells also require a chemical, mechanical or membrane receptor induced stimulus to differentiate to the correct phenotype. In this experiment the surface will be functionalized with the bidentate functional group. To one side of the functional group, peptides which have demonstrated an ability to bind cells such as the sequence RGD or YIGSR subunits will be attached. To the other terminal of the functional group we will attach growth factors which act through surface receptor mediated stimulations.

Surface will be created with either RGD peptide, fibroblast growth factor, both or bsa immobilized through the bidentate functional group. Surfaces will be seed with L929

mouse fibroblasts through both static or flow seeding. Surfaces will then be cultured for 7days and then analyzed for proliferation through the MTT assay, trypsinized to count the number of adhered cells and then isolated for RNA. Following isolation of RNA, cells will be analyzed by real time quantitative PCR to determine whether specific phenotype markers and ECM producing genes have been up-regulated in relationship to house keeping genes. Secondary experiments will be conducted with RGD peptides and vascular endothelial growth factor (VEGF) can increase the seeding, differentiation and proliferation of endothelial progenitor cells. Finally surfaces will be immobilized with YIGSR and VEGF and binding attempted with mesenchymal stem cells to determine if the surface can aid in proper differentiation of the stem cells. These surface will be analyzed for gene transcription as described above but also for proper blood flow over the material surface.

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