Methacrylic Terpolymer Biomaterials for Cardiovascular Applications

Dissertation

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By

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Preface

This dissertation is divided into seven chapters: Chapter 1 provides background information on the problem of biomaterial-induced thrombosis; Chapters 2 through 6 present original research conducted at The Ohio State University in an attempt to solve this important biomedical problem, and Chapter 7 supplies the reader with the overall conclusions of this research and suggestions on the direction my successors in The Cooper Group may take when continuing this work.

My hope is that the content of this dissertation will be of use to researchers interested in the structure-property relationships of polymer systems and for those in the biomaterials community who focus on tailoring the interfacial phenomena which occurs between materials and biology.

For the convenience of the reader, the sections of this document which discuss original research (Chapters 2 through 6) are designed as self-contained and independent units each containing an abstract, introduction, materials and methods, results and discussion, and conclusions section.
Abstract

This dissertation focuses on the development of a novel methacrylic terpolymer biomaterial system intended for use in small diameter vascular graft applications. The material is produced through the random copolymerization of hexyl methacrylate (HMA), methyl methacrylate (MMA), and methacrylic acid (MAA). Small amounts of MAA were incorporated (2 mol %) which enable post-synthesis derivatizations, if necessary. The remaining 98 mol % of the material is either HMA or MMA. By controlling the ratio of these two co-monomers materials with very different glass transition temperatures (T_g) – and thus very different physical properties – were generated. Human umbilical vein endothelial cells (HUVECs) are able to adhere, spread, and divide on the surface of the biomaterial illustrating its cytocompatibility.

These materials were then fabricated into three dimensional and porous scaffolds through electrospinning. Scaffolds composed of random fibers were generated by electrospinning onto a stationary collector while scaffolds composed of aligned fibers were generated by electrospinning onto a rotating collector. The degree of fiber alignment is influenced by the rotation rate of the collector. The morphology of the fibrous scaffolds is greatly influenced by the glass transition temperature of the polymer. Low T_g materials produce scaffolds with low porosity and fused fibers while high T_g materials produce scaffolds with high porosity and discrete fibers. Though all scaffolds adhere similar numbers of cells over short time spans, cells on the low porosity materials exhibit higher proliferation rates, higher enzymatic activity, and a more spread morphology in comparison to other three dimensional scaffolds and flat polymer films after
several days of culture. Though cells on the aligned scaffolds did not show increased proliferation they did acquire an elongated morphology more similar to what is observed in vivo.

Peptide ligands were incorporated into the polymer materials through chain transfer chemistry and the presence of the ligand was confirmed through amino acid analysis. Polymers where produced which contained several different polypeptide sequences: the RGD tripeptide unit which has been shown to bind to mature endothelial cells; novel peptides found through phage display technology which bind with high specificity to human blood outgrowth endothelial cells (HBOECs); and the non-adhesive RGE tripeptide unit which acts as a negative control. Fibronectin coated glass coverslips were used as the positive control. Increased attachment of mature endothelial cells was observed on the RGD-containing materials in comparison to the RGE-containing controls and up regulation of HBOEC adhesion was observed on materials functionalized with one of the novel peptide units in comparison to the RGE-control. However, these results were only observed when the cell adhesion assays were performed in serum free media possibly due to the adsorption of serum proteins to the interface which acted to shield the peptide ligands from the attaching cells.

In order to combat the non-specific adsorption of proteins the formulation of the polymer was changed. MAA was removed and replaced by a methacrylate monomer which contains a poly(ethylene oxide) pendant group (PEGMA). Materials polymerized from a feed containing 0 – 25 mol % PEGMA were synthesized and materials containing > 15 mol % exhibited excellent non-fouling interfacial properties as observed through resistance to fibrinogen adsorption, platelet attachment, and HUVEC adhesion. Being thermoplastic, these materials were fabricated into fibrous scaffolds through electrospinning and it was shown that scaffolds are also resistant to cellular attachment.
A second approach to combating non-specific interaction with the biological environment is through incorporating zwitterionic chemical groups. Therefore the PEGMA was removed and formulations polymerized from feeds containing 0 – 30 mol % sulfobetaine methacrylate (SBMA) were produced. It was found that materials polymerized from ≥ 15 mol % of the zwitterionic co-monomer showed strong resistance to biofouling as again measured through fibrinogen adsorption, platelet attachment, and HUVEC adhesion.
Dedication

Dedicated to all my mentors, past and present.

I could not have done this without you.
Acknowledgements

At some points during my graduate student career I worried that I was not talented or smart enough to complete a Ph.D. Luckily, whenever I doubted myself someone was always there to remind me of why I chose to pursue this degree and to encourage me to keep moving forward. The individuals listed below deserve special thanks because their encouragement and support made the completion of this degree possible.

First and foremost I need to thank my research advisor, Professor Stuart Cooper, who provided me a great project to work on, helped me struggle through difficult problems, was patient and understanding, and – most importantly – believed in me. Here’s to number 61 Professor Cooper!

My family and friends also deserve a special thank you. They were always a source of encouragement and support throughout my time at Ohio State.

Xin Wang, Ruth Li and all my other research collaborators were always available to listen to my ideas and provide constructive feedback. They helped me plan better experiments and thus improve the quality of this dissertation.

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Chapter 1: Background

1.1. Thrombus Occlusion of Small Diameter Vascular Grafts

Biomaterials are materials – natural, manmade, or composite – which are used in biomedical devices or in contact with biological systems and act to restore the body’s natural function.\(^1\)

Many clinically successful biomaterials exist today and are used in a wide array of biomedical devices such as joint replacements, bone cements and plates, blood vessel prosthesis, artificial heart valves, contact lenses, intraocular lenses, cochlear implants, breast implants, and drug delivery devices.\(^1\)

Several sources of replacement materials exist and each has its own unique sets of advantages and disadvantages. Using the patient’s own tissue – autografting – is often preferred. However, limited availability, second site morbidity, infection, and tendency towards resorption limit this approach.\(^1\)\(^3\) Similarly, the use of another person or animal’s tissue – allografting/xenografting – is limited by the need for immunosuppressant drugs, infection, and disease transfer.\(^1\)\(^4\) In many instances, biomaterials are the best option due to their availability, reproducibility of physical properties, and the elimination of disease transfer.\(^1\)\(^4\) However, synthetic biomaterials also have limitations such as poor integration with surrounding tissue, initiation of the foreign body response, poor blood compatibility, production of potentially harmful degradation products, and mechanical mismatch with adjoining tissue.\(^1\)\(^2\) Despite the success of many biomedical devices, these limitations clearly illustrate the need to develop new materials and technologies to achieve better device function and improved healing.
The work presented in this dissertation focuses on an important subclass of biomaterials, those used in blood-contacting applications. Examples of such cardiovascular biomedical devices include large and small diameter vascular grafts, artificial heart valves, total artificial heart, stents, and pacemaker lead insulation. Like all other areas of biomaterials, problems exist with current cardiovascular biomaterials. For instance, the polyurethane insulation on pacemaker leads is subject to metal induced oxidation from the metal leads resulting in undesired degradation of the insulation. However, the most ubiquitous problem with cardiovascular medical devices is biomaterial-induced thrombosis, or the formation of a blood clot at the blood-biomaterial interface. The presence of thrombus can either directly impair the function of the device (such as hindering the motion of artificial heart valve leaflets) or release thrombus particles – emboli – which can lodge downstream, interrupt blood flow, and cause tissue ischemia and necrosis.

Specifically, the novel biomaterial developed in this research is intended for use in small diameter vascular graft applications so it is fitting to discuss the history and current use of vascular prosthesis in medicine. The first vascular graft was made in 1952 by Voorhees from the fabric Vinyon N. Soon after poly(ethyelene terephthalate) (PET or Dacron®) woven grafts emerged as an excellent graft material. In fact, Dacron® grafts along with those made from expanded poly(tetrafluoroethylene) (ePTFE) are the two most common vascular graft materials in use today. Large diameter vascular grafts (with a diameter larger than 6mm) are clinically very successful with a patency rate of approximately 90% after 5 – 10 years. However, their small diameter counterparts (with a diameter less than 6mm) quickly lose patency. Less than 50% of small diameter vascular grafts remain functional after 5 years of implantation. Often small diameter vascular grafts fail due to thrombus which develops on the surface of the graft and eventually occludes the lumen resulting in loss of blood flow to downstream tissue. Occlusion of the vascular replacement often requires the patient to undergo subsequent surgeries and can result in amputation or patient death. Large diameter vascular grafts also develop thrombus;
however, graft occlusion often does not often occur due to the larger cross sectional area of the graft and the high shear in these vessels which results in stabilization of the thrombus coated graft\textsuperscript{1,8-10}.

Clearly the failure of small diameter vascular grafts by thrombus occlusion occurs due to the inadvertent activation of the body’s coagulation response. Therefore, it is logical to hypothesize that creating a surface which does not result in blood clotting could be used to improve the function of small diameter vascular replacements. However, before one could design such a material it is necessary to know what interactions occur between the blood and the biomaterial.

1.2. Tailoring the Interface of Blood-Contacting Biomaterials

The search for a truly blood compatible interface – which dates back over 50 years – has been long and the solution still eludes us today. However, finding a solution is highly significance to the medical community. To stress the importance of blood-material interactions Buddy Ratner wrote a manuscript in 1993 entitled \textit{The Blood Compatibility Catastrophe} which was meant to act as a "call to arm" to spur the development of a non-thrombogenic interface.\textsuperscript{11} However, in 2007 – nearly 15 years later – he published a companion article entitled \textit{The Catastrophe Revisited: Blood Compatibility in the 21st Century}, and in this article Ratner laments that vast improvement can still be made to many cardiovascular devices. For instance, recipients of a blood contacting biomedical device still require large amounts of anticoagulants (which are both expensive and pose high risk to the patient), no successful small diameter vascular grafts have been developed, and devices such as the extracorporeal membrane oxygenators cause large amounts of damage to blood.\textsuperscript{12}

Thrombosis is the process through which the body coagulates blood and the result of this process is thrombus, a blood clot.\textsuperscript{1,13} During instances of blood vessel injury, thrombosis is an important
mechanism the body uses to plug the wound, halt blood loss, and maintain hemostasis. For coagulation to occur platelets and blood components called coagulation factors must be activated and participate in what is called the coagulation cascade. First platelets adhere to the site of vessel injury and become activated. During this activation step they release chemical signals which activate other platelets in the blood stream and recruit them to the site of injury. Eventually enough platelets agglomerate in order to plug the wound. However, this plug is not very strong and could be dislodged and washed away easily. Therefore, coagulation factors crosslink the blood protein fibrinogen into fibrin which acts to reinforce and stabilize the clot.\textsuperscript{1,14-16} The coagulation cascade is vital to our survival. In fact, malfunctions in the clotting cascade are often diagnosed as potentially life threatening illnesses such as hemophilia (an inability to develop thrombus resulting in prolong bleeding) or thrombophilia (a predisposition to developing thrombus which can result in thromboembolism, tissue ischemia, and necrosis).\textsuperscript{17-22}

Though thrombosis is necessary to maintain hemostasis after vascular injury, the body also employs two regulatory systems to control clot size. The first mechanism uses coagulation inhibitors which interfere with the crosslinking of fibrinogen to fibrin which hinders or stops clot growth.\textsuperscript{23} The second mechanism is fibrinolysis in which proteases cleave fibrin resulting in clot degradation.\textsuperscript{24,25} When blood contacts a foreign surface, the situation is markedly different. Upon implantation, the biomaterial interface is immediately covered with a layer of proteins which adsorb from the bloodstream. These proteins mediate the adhesion of various cell types – including platelets – to the interface. These platelets are then activated into their pro-thrombogenic state and blood coagulation ensues.\textsuperscript{1,26,27} The above mentioned mechanisms for regulating clot size do not appear sufficient for controlling coagulation on implanted biomaterials.

Many attempts have been made to improve blood interactions with vascular grafts through various surface modifications.\textsuperscript{1} Most of these surface modifications can be classified as either biotolerant or bioactive. Biotolerant materials are designed to be inert in the body, meaning they
do not interact with platelets, coagulation factors, etc. Examples of biotolerant surface modification are hydrogel coatings and poly(ethylene glycol) (PEG) immobilization. Bioactive materials have biological cues built into the structure to produce blood compatibility through methods employed by nature. An example of a bioactive surface modification is heparinization.

In the above paragraphs we have discussed how blood clots on foreign surfaces. In fact, only one truly blood compatible surface has been discovered – the endothelium of native vasculature. All blood vessels are lined with a confluent monolayer of endothelial cells and actively work to prevent thrombus so researchers have also pre-seeded vascular grafts with endothelial cells prior to implantation which has improved the blood compatibility of the graft. However, this method requires isolation of the host’s endothelial cells and the pre-culturing of such cells on the material surface before implantation. Furthermore, the endothelium is delicate and often undergoes damage during implantation. Nevertheless, many researchers still believe that a confluent and functioning endothelial cell layer – mimetic of what is found in native vasculature – is the only truly blood compatible interface.

To facilitate endothelial cell attachment researchers have exploited the molecular biology of the cell membrane. The cell membrane is a complex structure composed mostly of phospholipid molecules. Interestingly, these amphipathic molecules are not held together by covalent linkages or long range molecular entanglements as most man-made products are. Rather, the cell membrane, a lipid-lipid bilayer, is held together by Van der Waals interactions. Though not mechanically strong, the self assembled structure imparts unique and beneficial functionality to the membrane such as efficient inhibition of unregulated molecular transport and a fluid-like behavior for easy adaptation of shape.
Embedded in the cell membrane are 6 super-families of adhesion receptors which play various roles in cell adhesion, migration, targeting, and inflammation.\textsuperscript{38-41} The integrin superfamily of receptors is responsible for most cell-environment attachments. These receptors are transmembrane proteins with $\alpha$ and $\beta$ subunits which bind with high specificity to glycoproteins found in the extracellular matrix. On the inside of the cell the integrin receptors are attached to the structurally strong cytoskeleton of the cell (actin filaments). Though matrix proteins are very large macromolecules (the molecular weight of fibronectin is approximately 500kDa), many share certain amino acid sequences in common which act as binding site – or ligands – for integrin mediated cell attachment.\textsuperscript{42} The most studied peptide ligand is the arginine, glycine, aspartic acid (RGD) tripeptide sequence which is found in fibrinogen, fibronectin, vitronectin, and von Willebrand factor.\textsuperscript{43-46} Other cell binding domains have been identified such as the tyrosine, isoleucine, glycine, serine, arginine (YIGSR) pentapeptide sequence found in laminin and the arginine, glutamic acid, aspartic acid, valine (REDV) tertapeptide which is also found in fibronectin.\textsuperscript{47-48}

There have been many studies of biomaterials functionalized with these adhesive peptide sequences which resulted in improved cellular attachment.\textsuperscript{48-50} Focusing on endothelial cells, the RGD tripeptide unit is most often used to up regulate cellular attachment since these cells bare many integrins capable of binding this ligand such as the $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ receptors.\textsuperscript{51} Yet even with these bio-inspired surfaces no blood compatible biomaterial has made it to market.

1.3. Our Proposed Solution

Three methods of vascular graft endothelialization are seen \textit{in vivo}: tissue overgrowth, angiogenesis, and attachment of circulating cells.\textsuperscript{1} When a surgeon implants a graft into a patient it is sutured to a native vessel. As part of the healing process, tissue from the viable blood vessel
grows axially onto the edges of the graft. This growth is common; however, it generally does not spread beyond the region close to the graft/vessel interface. If a graft is implanted in close proximity to a native blood vessel angiogenesis (the generation of new blood vessels) will sometimes occur. If the graft is processed with proper porosity, these new vessels can penetrate the graft and the endothelial cells will begin to spread on the luminal surface. Endothelial cells can also be found circulating freely in the blood stream. If one of these circulating cells is transported to the graft surface and attaches it can divide and endothelialize the graft in the location immediately surrounding the attachment site.\(^1\) The three methods of endothelialization are shown schematically below in Figure 1.1. Many animal species can fully endothelialize vascular graft materials; however, this behavior is not seen in humans. Furthermore, the attachment and division of circulating cells has been implicated as the primary method which leads to this endothelialization.\(^1\)

![Figure 1.a](image1.png)

![Figure 1.b](image2.png)

![Figure 1.c](image3.png)

**Figure 1.1:** Methods of graft endothelialization. (a) Tissue overgrowth from adjacent blood vessel, (b) luminal endothelialization of graft from angiogenesis of adjacent blood vessels through graft pores, (c) endothelialization through the attachment and growth of circulating endothelial cells.
Though mature endothelial cells are found in circulation, in 1997 Asahara and colleagues published an article in *Science* describing the isolation of a subpopulation of CD34+ adult stem cells from peripheral blood which possessed the capacity to differentiate into cells with endothelial phenotype. These cells were termed endothelial progenitor cells (EPCs). Furthermore, the outgrowth from these cells were found to have higher proliferation capacities and rates in comparison to mature endothelial cells making them an attractive cell source for the endothelialization of medical devices. One of the first attempts to employ EPCs in the endothelialization of small diameter vascular grafts was published in *Circulation* in 2005 by Rotmans and coworkers. In this work an expanded poly(tetrafluoroethylene) graft was modified with anti-CD34 antibodies and implanted into a porcine model as an arteriovenous shunt in an attempt to scavenge these adult stem cells from circulation and endothelialize the interface. Although the grafts did adhere a large population of cells which were positively identified as endothelial cells by lectin staining a 3 fold increase in the failure of anti-CD34 modified grafts was observed due to neointimal hyperplasia at the venous anastomosis in comparison to unmodified grafts. Two possible explanations were given for the stimulation of neointimal hyperplasia in the modified grafts: 1) EPCs have the capacity to differentiate into vascular smooth muscle cells which could have promoted the hyperplasia, and 2) EPCs produce potent growth factors which could have spurred on vascular smooth muscle cell proliferation at the venous anastomosis. Though this study did not provide a method for creating a clinically successful vascular graft it clearly illustrates the potential for using EPCs as a means of developing an endothelium on biomedical devices.

Subsequently EPCs have been isolated from peripheral blood and cultured *ex vivo*. The *ex vivo* expansion of EPCs yields two very different populations of cells: early outgrowth EPCs and late outgrowth EPCs. Early outgrowth cells have a spindle-like morphology and release more angiogenic cytokines such as VEGF while late outgrowth cells (after 2-3 weeks of culture) have a
cobblestone-like morphology and demonstrate more endothelial-like behavior (greater NO production and incorporation into endothelial networks). However, these late outgrowth endothelial cells are still distinct from mature endothelial cells in that they possess much greater proliferation rates and capacities.\textsuperscript{55}

Late outgrowth EPCs from human peripheral blood are also termed human blood outgrowth endothelial cells (HBOECs). We feel that these cells are an attractive source for the endothelialization of medical implants since they couple endothelial-like phenotype with higher proliferation capacities and rates. Therefore, we wish to create a material tuned to the specific adhesion and growth of HBOECs and hypothesize that such a surface would be able to generate a confluent and functioning endothelium which does not promote neointimal hyperplasia since these cells are committed to their endothelial phenotype and produce fewer growth factors than early outgrowth cells.\textsuperscript{55}

The work presented in this dissertation describes the development of a novel methacrylic biomaterial with tunable mechanical properties. The interface of the materials is then altered through various fabrication techniques and chemical and biological functionalizations in order to optimize the surface for endothelialization and the specific adhesion and growth of HBOECs.
Chapter 2: Design and Characterization of the Methacrylic Terpolymer System for Cardiovascular Biomaterial Applications

2.1. Abstract

A biomaterial system with tunable mechanical properties was synthesized through random copolymerization of hexyl methacrylate, methyl methacrylate, and methacrylic acid. The composition, molecular weight, water absorption, glass transition temperature, and tensile properties of the polymer system were characterized, and the biocompatibility of the material was demonstrated by the ability of human umbilical vein endothelial cells to adhere, proliferate, and spread on the polymer surface. No difference was observed in the adhesion and growth of the endothelial cells on the materials regardless of modulus; however, cells on the tissue culture polystyrene positive control showed enhanced cell spreading.

2.2. Introduction

In this chapter we focus on biomaterial mechanical properties, which can play an important role in a biomaterial’s success both on a tissue level and a cellular level. When two mechanically dissimilar materials are joined and then deformed, stresses develop at the interface. At a tissue level, when the two mechanically dissimilar materials are an implant and host tissue, sub-optimal healing or implant failure often result. For instance, many ceramics and glasses cannot be used clinically to repair defects in load-bearing bones (femur, tibia) due to low fracture toughness while...
metal implants can retard bone remodeling and increase bone porosity due to the metal’s much higher elastic modulus.²⁻⁴ Mechanical mismatch with urinary, ligament, and cartilage tissue has resulted in poor healing and tissue erosion, and compliance mismatch between vascular grafts and arterial tissue is believed to play a role in the development of thrombus and neointimal hyperplasia.⁵⁻¹⁰ The stiffness of a biomaterial is also important on a cellular level as it has been shown to control the behavior of adherent cells.¹¹⁻¹³ A potent example of how biomaterial stiffness affects cell behavior is seen with valvular interstitial cells (VICS) which are fibroblasts present in heart valves. Though these cells have great potential in the tissue engineering of heart valves, scaffold stiffness is of chief concern. When cultured on materials with a modulus > 15 kPa they undergo activation into a myofibroblast state and calcify their environment which would lead to valve sclerosis.¹⁴⁻¹⁵

In this work, we wished to create a polymeric and cytocompatible biomaterial system with tunable mechanical properties through a copolymerization technique. Successful synthesis of such a biomaterial would allow a single polymer system to be used for a variety of biomaterial applications, and will allow us to probe how stiffness affects the behavior of adherent endothelial cells. The methacrylate family of polymers was selected for this research due to its long history of use in the medical field. Methacrylates have been employed commercially in devices such as bone cement and hard and soft contact lenses.²,¹⁶⁻¹⁸ The material studied in this research is a terpolymer produced by free radical copolymerization of hexyl methacrylate (HMA), methyl methacrylate (MMA), and methacrylic acid (MAA).¹⁹ The resulting polymers are random and atactic, and as a result of the irregularity of the terpolymer backbone, the material is fully amorphous. Through variation of the molar concentration of HMA (a soft and viscoelastic homopolymer) and MMA (a glassy and brittle homopolymer) incorporated into the polymer one has a high degree of control over the physical behavior of the resulting material through specification of the glass transition temperature.²⁰ The molar concentration of MAA incorporated into the material was held constant at 2 mole % which enables post-synthesis derivitizations such
as ionic crosslinking with metal salts and incorporation of biofunctional molecules.\textsuperscript{19-21} The material has been shown to have excellent \textit{in vitro} stability in hydrolitic and oxidative environments by virtue of its saturated carbon-carbon backbone making it ideal for applications where long term maintenance of physical properties is necessary.\textsuperscript{22} The target application for this polymer system is for small diameter vascular grafts where the biostability of the material will allow problems such as aneurysm and rupture of the graft to be avoided.

2.3. Materials and methods

\textbf{Polymer synthesis.} The terpolymer was produced through free radical polymerization in 100 mL of N,N-dimethylformamide (DMF). The solvent was purged with argon for a minimum of 2 hours to remove oxygen. 20 g of the desired monomer mixture was added to the reaction vessel and purged for an additional 1.5 hours. The temperature was increased to 55 – 60 °C and 0.0040 g of 2,2-azobisisobutyronitile (AIBN) free radical initiator was added to the system. The reaction was performed under reflux and was allowed to proceed for 48 hours. Upon termination of the reaction, the polymer was precipitated into an equivolume mixture of methanol and distilled water. The precipitated polymer was collected, dried, and weighed.

\textbf{Polymer composition.} The composition of the terpolymer was determined through a combination of \textsuperscript{1}H Fourier transform nuclear magnetic resonance (NMR) spectroscopy and acid-base titration. 600 μL of a 0.03 g/mL polymer solution in deuterated chloroform was transferred to a Wilmad NMR sample tube (Warminster, PA). The NMR analysis was performed on a DMX 600 MHz NMR machine at 300 K. 128 scans were used to construct the NMR spectra. A peak at 4.1 ppm represents the two protons adjacent to the oxygen atom in the HMA pendant group and a peak at 3.8 ppm represents the three protons present in the terminal methyl group of the MMA pendant group. The molar ratio of HMA to MMA in the polymer was determined by taking the integral of the peak at 4.1 ppm and dividing it by 2 and the peak at 3.8 ppm by 3. The MAA
comonomer was incorporated at low levels (2 mole %) and the peak was not visible in the NMR spectra. Therefore, acid-base titration was used to quantify the presence of the acid repeat unit.

For the acid-base titration an equivolume mixture of toluene and ethanol was used as solvent. The titrant was a 0.1g/L solution of NaOH in distilled water. The mass of an approximately 0.1 g polymer sample was accurately measured and recorded using a Mettler AE163 analytical balance. 50 mL of the solvent was added to an Erlenmeyer flask containing the terpolymer and after dissolution 3 drops of phenolphthalein were added. The solution was titrated until a faint pink color was observed and persisted. The volume of titrant used to neutralize the polymer solution was recorded. An Erlenmeyer containing no polymer was also titrated in order to correct for the acidification of the solution due to exposure to the atmosphere.

All polymer characterization included tests of three different batches of terpolymer at each composition level so these data include batch-to-batch variations. Thus molecular weight, composition, water absorption, and tensile measurements were repeated in triplicate for each material batch.

**Molecular weight.** Gel permeation chromatography was performed at 35°C using a Waters Breeze system equipped with a Waters 410 refractive index detector and a Waters Styragel column (Milford, MA). Tetrahydrofuran (THF - HPLC grade) (Sigma-Aldrich) was used as the eluent at 1.0 mL/min. The sample concentration was between 1 – 5 mg of polymer/mL THF. The injection volume was 100 µL. The equipment was calibrated using monodisperse polystyrene standards.

**Water absorption.** Polymer films were cast from solution to remove any topography which could hold bulk water. Briefly, 4 g of polymer was dissolved in 40 mL of THF. The solution was poured into a 100 mL teflon Petri dish, covered with aluminum foil, and allowed to slowly dry over night.
The film was then dried in a convection oven at 55 – 60°C for 24 hours and further dried in a vacuum oven at 55 – 60°C for 24 hours to remove residual solvent. Upon removal from the vacuum oven, the polymer film was cut into samples weighing approximately 0.2 g. The dry mass of the sample was measured and recorded and the samples were submerged in deionized water. The samples were removed from water, patted dry using a lint free cloth, and weighed after 24 hours and every 2 days for 2 weeks. The percent change in mass was calculated to characterize the water uptake properties of the material. The percent change in mass after 2 weeks of submersion was taken as the equilibrium value.

**Glass transition temperature.** Solution cast polymer films were created as described above. Test samples were cut from the films using an ASTM D-638-5 die. The test samples were mounted in tension clamps and an RSA3 dynamic mechanical analyzer was used to measure the viscous (loss) modulus of the test samples. The temperature was ramped from -50 °C to 30 °C above the expected glass transition temperature, strained 0.1% with a frequency of 1 Hz, a temperature step of 3 °C/min, and a 20 second soak time. The glass transition temperature was determined as the temperature where the viscous modulus reached a maximum and began to decrease.

**Stress strain properties.** Tensile testing was performed in accordance with the ASTM D-638 protocol (Standard Test Method for Tensile Properties of Plastics). Tensile specimens were created as described in section 2.5 using an ASTM D-638-5 die. The dog bone shape samples had a total length of 63.5 mm and a gauge length of 7.62 mm. The test was performed on a table model Instron at ambient conditions with a deformation rate of 10 mm/min.

**Preparation of cell culture surfaces.** In these experiments polymer of a given composition was selected from one of the polymerization batches. 18mm pre-cleaned glass coverslips were dip coated into a 10 % (g/mL) solution of polymer in acetone. The coverslips were then dried in a
convection oven for 24 hours at 55 – 60°C, and then dried in a vacuum oven for 24 hours at 55 – 
60°C to remove residual solvent. Polymer coated glass coverslips were placed in the wells of a 
12 well plate. Wells were sterilized through exposure to 100% ethanol and UV radiation for 30 
minutes. Wells were rinsed three times with phosphated buffer saline (PBS) to remove residual 
ethanol and equilibrated overnight in PBS.

Cell culture. Human umbilical vein endothelial cells (HUVECs) were obtained from American 
Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured according to standard 
procedures. Briefly, the base media was Ham’s F12K media. The media was supplemented with 
0.1 mg/mL heparin (Sigma-Aldrich), 0.05 mg/mL endothelial cell growth supplement (Sigma-
Aldrich), and 10 volume % fetal bovine serum. Cultures were incubated in a humidified 
environment with 5% CO₂ at 37°C. The culture medium was changed every two days and 
cultures were passaged at 80% confluence to prevent contact inhibition. Passages 4 through 8 
were used. For cell experiments, HUVECs were lifted using 0.25 trypsin/EDTA, counted with a 
hemocytometer, centrifuged, old media was aspirated, and cells were diluted to the desired 
density through the addition of fresh media.

Cell adhesion and proliferation. 1 mL of media containing 50,000 cells was added to each well 
(13,000 cells/cm²) and incubated for the desired length of time. Unattached cells were removed 
with 3 washes with pre-warmed Dubelcco’s phosphated buffer saline (DPBS). The test surfaces 
were then transferred to a new well plate and covered with 1 mL of fresh media. Adherent cells 
were lysed using 3 cycles of freezing and thawing followed by sonication. 0.25 mL of a 5ug/mL 
solution of Hoechst 33258 was added to each well and allowed to incubate for 30 minutes under 
ambient conditions. 0.25 mL of cell lyses from each well was then transferred to a well of a 96 
well plate. Adherent cell numbers were determined by comparing the fluorescent intensity of 
each well to a calibration curve generated from a known number of cells. Cell densities were
calculated by dividing the cell number by the area of the test surface. Experiments were performed in triplicate and the fluorescence for each well was measured three times.

**Cell viability.** 1 mL of media containing 50,000 cells was added to each well (13,000 cells/cm$^2$) and incubated for the desired length of time. Unattached cells were removed using 3 washes with pre-warmed DPBS. The test surfaces were then transferred to a new well plate. The adherent cells were lifted through the addition of 200 μL of 0.25 trypsin/EDTA, and the cells were stained through the addition of 200 μL trypan blue. 10 μL of the cell suspension was transferred into a hemocytometer and the total number of cells and the number of non-viable (dark blue) cells were counted and used to calculate percent viability. Experiments were performed in triplicate and viability was calculated for each surface 4 times.

**Cell morphology.** Cell morphology was assessed under sparsely seeded and nearly confluent conditions. In each case, 1 mL of cell suspension was added to each well and incubated for the desired length of time. Unattached cells were removed using 3 washes with pre-warmed DPBS. Cultures were fixed in 4% paraformaldehyde for 30 minutes. Cells were permealized with 0.2% Triton for 5 minutes and then stained with DAPI and Alexa Fluor-488 phalloidin according to manufacturer instructions. Cultures were mounted using ProLong antifade reagent. Culture surfaces were then imaged using fluorescence microscopy at 10x and 20x magnification. For sparsely seeded conditions, 10,000 cells were added per well (2,600 cells/cm$^2$) and allowed to attach for 2 days. 15 randomly selected and non-overlapping images were taken on each test surface and the area occupied by the adherent cells was measured using ImageJ image analysis software. For nearly confluent conditions, 50,000 cells were seeded (13,000 cells/cm$^2$) and allowed to attach and grow for 15 days. The average area occupied by the adherent cells was determined through reciprocating the adherent cell density calculated in section 2.9.
Statistical analysis. All values are reported as means ± standard deviations. When comparing multiple means, an ANOVA was used to determine if any of the pairs showed statistical difference and a Tukey-Kramer Honest Difference Test was used to determine which of the pairs were statistically different. In all tests $\alpha < 0.05$.

2.4. Results and Discussion

2.4.1. Polymer characterization

Polymer composition. Figure 2.1 contains a schematic of the polymer chains synthesized in this study. The terpolymer is referred to by the mole percent of hexyl methacrylate used in its synthesis. For instance, H90 refers to a material synthesized from 90/8/2 mole % HMA/MMA/MAA. The yield from the polymerization reactor after 48 hours was approximately 50% regardless of polymer composition. The yield of the reactor could be increased by either allowing the reaction to continue for longer or by adding additional initiator. However, using a higher concentration of initiator would result in a decrease of polymer molecular weight. Since these materials are atactic and therefore non-crystalline and the polymer chains are relatively non-polar, high molecular weight was needed to ensure the necessary physical properties of the polymer.
As seen in Table 2.1 the composition of the polymer is similar to the initial charge of the reactor; however, at all composition levels we see an accumulation of HMA in the polymer. Two possible explanations for this observation are (1) the effect of reactivity ratios and (2) monomer volatility. The reactivity ratio is a concept in copolymerization theory which describes the propensity of a radical species to add to a monomer of the same species as itself versus a different species. The accumulation of HMA in the polymer could be due to its preferential addition compared to MMA. MMA is also the most highly volatile of the monomers. Though the reaction takes place under reflux, it is possible that some of the monomer is evaporating and exiting the system along with the argon gas feed resulting in a decrease in the feed concentration of MMA and an enrichment of HMA in the polymer. Despite the slight enrichment of HMA seen in the material, polymer with a wide range of compositions has been generated. Furthermore, the standard deviations associated with the data are small illustrating the reproducibility of the synthesis process. It is
important to note that the presence of the MAA in the polymer backbone could cause unwanted calcification of the implant over time. However, in future work these acid sites will most likely be removed through post-synthesis functionalization such as the incorporation of cell adhesive peptide ligands.

<table>
<thead>
<tr>
<th>Terpolymer (mol % HMA)</th>
<th>Reactor composition (mol % HMA/MMA/MAA)</th>
<th>Polymer composition (mol % HMA/MMA/MAA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10</td>
<td>10/88/2</td>
<td>12/85/2 ± 2/2/0</td>
</tr>
<tr>
<td>H20</td>
<td>20/78/2</td>
<td>24/74/2 ± 3/3/0</td>
</tr>
<tr>
<td>H30</td>
<td>30/68/2</td>
<td>35/63/2 ± 2/2/0</td>
</tr>
<tr>
<td>H40</td>
<td>40/58/2</td>
<td>45/54/2 ± 2/2/0</td>
</tr>
<tr>
<td>H50</td>
<td>50/48/2</td>
<td>55/43/2 ± 2/2/0</td>
</tr>
<tr>
<td>H60</td>
<td>60/38/2</td>
<td>66/33/1 ± 1/1/0</td>
</tr>
<tr>
<td>H70</td>
<td>70/28/2</td>
<td>74/24/1 ± 0/0/0</td>
</tr>
<tr>
<td>H80</td>
<td>80/18/2</td>
<td>84/15/1 ± 1/1/0</td>
</tr>
<tr>
<td>H90</td>
<td>90/8/2</td>
<td>92/7/1 ± 0/0/0</td>
</tr>
</tbody>
</table>

Table 2.1. Terpolymer composition versus reactor feed composition.

**Molecular weight.** Through analysis of the GPC spectra, the number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI) of the terpolymer were calculated and are reported in Table 2.2. The molecular weight averages are independent of the terpolymer composition with all number average molecular weights at approximately 230 KDa. Also, the polydispersity is low indicating that the spread of molecular weights produced in the polymerization reaction was small.
<table>
<thead>
<tr>
<th>Terpolymer (mol % HMA)</th>
<th>$M_n$ (KDa)</th>
<th>$M_w$ (KDa)</th>
<th>PDI (--)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10</td>
<td>240 ± 25</td>
<td>262 ± 21</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>H20</td>
<td>223 ± 15</td>
<td>247 ± 13</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>H30</td>
<td>257 ± 12</td>
<td>272 ± 8</td>
<td>1.06 ± 0.02</td>
</tr>
<tr>
<td>H40</td>
<td>228 ± 15</td>
<td>251 ± 13</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>H50</td>
<td>231 ± 27</td>
<td>253 ± 20</td>
<td>1.10 ± 0.04</td>
</tr>
<tr>
<td>H60</td>
<td>232 ± 16</td>
<td>253 ± 12</td>
<td>1.09 ± 0.03</td>
</tr>
<tr>
<td>H70</td>
<td>218 ± 20</td>
<td>244 ± 15</td>
<td>1.12 ± 0.00</td>
</tr>
<tr>
<td>H80</td>
<td>230 ± 22</td>
<td>255 ± 20</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td>H90</td>
<td>220 ± 7</td>
<td>246 ± 4</td>
<td>1.12 ± 0.02</td>
</tr>
</tbody>
</table>

Table 2.2. Terpolymer number average molecular weight ($M_n$), weight average molecular weight ($M_w$), and polydispersity index (PDI).

Water absorption. Implanted biomaterials will be located in a highly hydrated environment; therefore, it is necessary to characterize how the materials swell in the presence of water. HMA and MMA have little polarity and no ionic character resulting in hydrophobic homopolymers while MAA’s carboxyl group produces a hydrophilic homopolymer. Since the MAA is incorporated in low amounts (2 mole %) the terpolymer studied in this research is hydrophobic as observed in Table 2.3. However, the equilibrium water absorption observed (0.6 – 2 wt %) is higher than the reported equilibrium water absorption value of poly(methyl methacrylate) of 0.3 wt%.$^2$ This can be rationalized by the presence of the carboxylic acid groups which increased the hydrophilicity of the polymer system.
### Table 2.3. Terpolymer equilibrium water absorption.

<table>
<thead>
<tr>
<th>Terpolymer</th>
<th>Equilibrium water absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mol %HMA)</td>
<td>(weight %)</td>
</tr>
<tr>
<td>H10</td>
<td>2.00 ± 0.73</td>
</tr>
<tr>
<td>H20</td>
<td>1.25 ± 0.93</td>
</tr>
<tr>
<td>H30</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>H40</td>
<td>0.92 ± 0.43</td>
</tr>
<tr>
<td>H50</td>
<td>0.70 ± 0.50</td>
</tr>
<tr>
<td>H60</td>
<td>0.60 ± 0.28</td>
</tr>
<tr>
<td>H70</td>
<td>0.91 ± 0.33</td>
</tr>
<tr>
<td>H80</td>
<td>1.42 ± 0.40</td>
</tr>
<tr>
<td>H90</td>
<td>0.97 ± 0.22</td>
</tr>
</tbody>
</table>

**Glass transition temperature.** The glass transition temperature \((T_g)\) occurs when the polymer chains obtain enough thermal energy to begin long-range backbone rotation. Macroscopically, this is observed as the softening point of the material. Poly(methyl methacrylate) has a \(T_g\) of 105°C while poly(hexyl methacrylate) has a much lower \(T_g\) of -5°C due to its long, linear, and non-polar pendant group. Through random copolymerization of the monomers, the glass transition temperature can be specified by controlling the ratio of HMA and MMA incorporated into the polymer chains. Initially dynamic scanning calorimetry (DSC) was used to determine the glass transition of the copolymer. However, the change in slope in the DSC spectra which indicates the glass transition was too broad and shallow to allow an accurate measurement of the \(T_g\). Therefore, dynamic mechanical analysis (DMA) was used to identify the transition temperature. Figure 2 shows representative tensile loss modulus \((E'')\) versus temperature profiles for the terpolymer system. The increased motion of the polymer chains when environmental conditions exceed the material's glass transition temperature can be observed as a decrease in the resistance to flow. Visually, this is the temperature in the DMA spectra where the viscous modulus goes through a maximum and begins to decrease. The representative DMA curves in Figure 2.2 illustrate that as the methacrylic terpolymer becomes richer in MMA the glass transition temperature increases.
Figure 2.2. Representative viscous (loss) modulus versus temperature profiles for the H20, H60, and H90 methacrylic terpolymer. The curves are shifted vertically to separate the data. The vertical line is an estimate of the $T_g$ of the material.

The Fox Equation (Equation 1) is an empirical equation used to estimate the glass transition temperature of random copolymers where $T_g$ is the glass transition of the copolymer, $T_{g,i}$ is the glass transition temperature of homopolymer $i$, and $w_i$ is the weight fraction of monomer $i$ in the copolymer.\textsuperscript{20}

$$\frac{1}{T_g} = \sum_i \frac{w_i}{T_{g,i}}$$  \hspace{1cm} (1)
Figure 2.3 compares the experimental data to the values predicted from the Fox Equation. As seen in the Figure, the Fox Equation underestimates the magnitude of the $T_g$ for the H20 to H60 terpolymers. Since the Fox equation is empirical it only provides a rough estimate of the $T_g$. For instance, the model assumes complete randomization of the order of repeat units in the backbone and cannot accurately predict the transition in a polymer where “blockiness” may be present. For instance, blocks of methyl methacrylate repeat units in the copolymer backbone could result in the discrepancy between the experimental and predicted glass transition values.

![Figure 2.3. Experimental and predicted values of the terpolymer glass transition temperature.](image-url)
**Stress strain properties.** In this section, we describe how several key physical properties change with the polymer composition: modulus, ultimate tensile strength (UTS), stress at break, and percent elongation. Figure 2.4 shows representative stress versus strain curves for the terpolymer over a range of polymer compositions. The tensile properties of the terpolymer system are compiled in Table 2.4. These data illustrate the wide range of physical behaviors which can be obtained from this polymer system. The goal of this research was to create a cytocompatible polymer with tunable mechanical properties through control of the glass transition temperature. As illustrated in the graph in Figure 2.4, the MMA-rich materials display the qualities of a glass such as high moduli and ultimate strengths yet relatively small percent elongations. As the materials becomes more rich in HMA the polymer takes on the macroscopic character of a rubber, moduli and ultimate strength decrease and large percent elongations are observed. The tensile strength of the coronary artery has been reported as 1.40 - 11.14 MPa with an ultimate strain of 45 - 99 %.\textsuperscript{23} These reported values are within the range of physical behaviors achieved with this polymer system.
Figure 2.4. Representative stress-strain behavior of the (A) H10, (B) H50, (C) H60, and (D) H90 methacrylic terpolymers.

<table>
<thead>
<tr>
<th>Terpolymer (mol % HMA)</th>
<th>Modulus (MPa)</th>
<th>UTS (MPa)</th>
<th>Stress at break (MPa)</th>
<th>Elongation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10</td>
<td>504 ± 73</td>
<td>44 ± 4</td>
<td>43 ± 3</td>
<td>14 ± 0.56</td>
</tr>
<tr>
<td>H20</td>
<td>438 ± 24</td>
<td>45 ± 6</td>
<td>44 ± 7</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>H30</td>
<td>295 ± 46</td>
<td>32 ± 2</td>
<td>32 ± 2</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>H40</td>
<td>346 ± 39</td>
<td>34 ± 2</td>
<td>28 ± 11</td>
<td>71 ± 101</td>
</tr>
<tr>
<td>H50</td>
<td>214 ± 41</td>
<td>24 ± 2</td>
<td>12 ± 2</td>
<td>220 ± 49</td>
</tr>
<tr>
<td>H60</td>
<td>74 ± 56</td>
<td>10 ± 2</td>
<td>9 ± 2</td>
<td>976 ± 100</td>
</tr>
<tr>
<td>H70</td>
<td>18 ± 14</td>
<td>4 ± 1</td>
<td>4 ± 1</td>
<td>1336 ± 134</td>
</tr>
<tr>
<td>H80</td>
<td>15 ± 5</td>
<td>2 ± 0.69</td>
<td>2 ± 0.70</td>
<td>1880 ± 162</td>
</tr>
<tr>
<td>H90</td>
<td>3 ± 2</td>
<td>0.80 ± 0.04</td>
<td>0.76 ± 0.04</td>
<td>1651 ± 201</td>
</tr>
</tbody>
</table>

Table 2.4. Terpolymer tensile properties: modulus, ultimate tensile strength, stress at break, and percent elongation.
2.4.2. Cellular interactions

Since the material is intended for use in cardiovascular applications, human umbilical vein endothelial cells (HUVECs) were used as the model cell. Cytocompatibility was probed by the ability of HUVECs to adhere, remain viable, and spread on terpolymer surfaces in static \textit{in vitro} culture. The test surfaces included the H20, H60, and H90 terpolymers and tissue culture polystyrene (TCPS) as the positive control. Cells were allowed to adhere under several conditions: 4 hours without serum, 4 hours with serum, and 2 days with serum, 5 days with serum, and 15 days with serum. The ability of a biomaterial to adhere a viable population of the desired cell type is an important step leading to acceptance of a biomaterial and integration with the biological system. Figures 2.5 and 2.6 illustrate the cytocompatibility of the polymer system by the ability of the HUVECs to adhere, proliferate, and remain viable on the polymer surface. Furthermore, these data indicate that the mechanical properties of the material can be changed with limited affect on the ability of HUVECs to inhabit the surface. The cytocompability of the polymer is further supported by the fluorescent images of Figure 2.7 which show a confluent endothelium on the polymer surface after 2 weeks of culture.

**Adhesion, proliferation, and viability.** The cell adhesion and proliferation profile of HUVECs on terpolymer substrata is presented in Figure 2.5. From the figure we see that cells are capable of adhering to and proliferating on the terpolymer surfaces. Furthermore, the cell density is largely independent of polymer composition. After 5 days in culture all surfaces develop a nearly confluent monolayer of endothelial cells, and by 15 days in culture no statistical difference is seen between the adherent cell density on terpolymer surfaces and the TCPS positive control indicating that all surfaces are capable of fostering an confluent monolayer of endothelial cells (as observed through fluorescence microscopy shown in section 3.2.2). Also, all surfaces were able to support at highly viable population of cells (>90% viability) regardless of chemical composition.
or time in culture as shown in Figure 2.6. Furthermore, no statistical difference was seen between the terpolymer surfaces and the TCPS illustrating that the terpolymer is not cytotoxic.

Figure 2.5. Adhesion and proliferation profile of HUVECs on terpolymer surfaces. The letters located within the bars denote statistical differences between these data. Bars which do not contain the same letter are statistically different.
Figure 2.6. Viability of HUVECs on terpolymer surfaces.

**Morphology.** For the 2 day cell morphology study, cells were sparsely seeded so the interaction between the biomaterial surface and individual cells could be assessed. Figure 2.7 (panels A, C, E, and G) shows individual cells adhering and spreading on the surface of the biomaterials. From the fluorescence microscopy images, the areas covered by the adherent cells were measured using ImageJ software. A threshold value of 1000 μm² was applied to the data. Cells covering more than this area were classified as spread. The morphology data are shown in Table 2.5. A large percentage of cells (>80%) were able to spread on the polymer surfaces after 2 days of culture. However, the 2 day cell spreading data was analyzed further by producing histograms of cell area on each surface. From the graphs presented in Figure 2.8 we can see that after 2 days of culture cells on the TCPS surface showed enhanced spreading (as illustrated by the shift of the histogram maximum to the right). This indicates potential exists for improving the surface of the terpolymer to enhance endothelialization. For instance, in future work we will incorporate surface
topography and peptide ligands with the hope of improving the adhesion and spreading of the adherent cells. Though after 2 days differences were observed between the biomaterials surfaces, after 15 days in culture a confluent endothelial monolayer of spread cells was obtained on all test surfaces as illustrated in Figure 2.7 (panels B, D, F, and H). From the cell density values calculated in 3.2.1 the average area covered by each cell is approximately 4000μm²/cell.
Figure 2.7. Fluorescent images of cells adherent to biomaterial surfaces: Panels (A) H20, (C) H60, (E) H90, and (G) TCPS are low seeding density after 2 days in culture at 20x magnification; Panels (B) H20, (D) H60, (F) H90, and (H) TCPS are seeded at high seeding densities after 15 days in culture at 10x magnification.
<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Spread cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>(%)</td>
</tr>
<tr>
<td>H20</td>
<td>85 ± 9</td>
</tr>
<tr>
<td>H60</td>
<td>80 ± 13</td>
</tr>
<tr>
<td>H90</td>
<td>80 ± 9</td>
</tr>
<tr>
<td>TCPS</td>
<td>96 ± 1</td>
</tr>
</tbody>
</table>

Table 2.5. Percent of spread cells after 2 day of culture in serum enriched media.

Figure 2.8. Histogram of HUVEC cell spreading after 2 days of culture in serum enriched media.
2.5. Conclusions

A methacrylic polymer system with tunable mechanical properties was designed through modulation of the glass transition temperature by varying the ratio of hexyl methacrylate and methyl methacrylate incorporated into the polymer backbone. The cytocompatibility of the material was illustrated through the ability of human umbilical vein endothelial cells to adhere, proliferate, and spread on the polymer surface. Interestingly, minimal difference was seen in the HUVEC interactions with materials with different stiffnesses. However, cell spreading on the tissue culture polystyrene positive control surface was greater than on the methacrylic terpolymers illustrating that improvements can be made to the terpolymer interface to enhance endothelialization.
Chapter 3: Electrospun scaffold topography affects endothelial cell proliferation, metabolic activity, and morphology

3.1. Abstract

A family of methacrylic terpolymer biomaterials was electrospun into three dimensional fibrous scaffolds. The glass transition temperature of the polymer influences the morphology of the resulting scaffold. Glassy materials produce scaffolds with discrete fibers and a high percent void space (84%) while the rubbery materials produced scaffolds with fused fibers and a much lower percent void space (18%). By electrospinning onto a rotating mandrel, aligned fiber scaffolds were fabricated, and it was shown that controlling the rotation speed of the collector allowed for control over the degree of fiber alignment. The electrospinning was shown to not degrade the number average molecular weight of the polymer chains. Human umbilical vein endothelial cells were seeded onto the electrospun scaffolds under static conditions and it was found that the morphology of the scaffold controlled the cellular proliferation, metabolic activity, and morphology of adherent cells. In particular, HUVECs seeded onto low void space scaffolds exhibited enhanced cellular spreading, enzymatic activity, and proliferation. HUVECs seeded onto aligned fiber scaffolds did not demonstrate increased proliferation; however, the cells did organize themselves in the direction of fiber alignment resulting in cells with elongated morphology.

3.2. Introduction

In most tissues, cells reside within or on top of a hydrated fibrous network called the extracellular matrix (ECM). ECM is chiefly composed of collagen and elastin fibers and an interfibrillary matrix
of glycoproteins and proteoglycans. The ECM is produced and maintained by the cells which
reside within it, and in turn the ECM provides many important functions such as mechanical
support for cell anchorage, determination of cellular orientation, maintenance of cell
differentiation, establishment of tissue microenvironment, and control over cellular adhesion and
migration.¹

ECM fibers are often narrower in diameter than the diameter of the adherent cells. Advances in
micro- and nano-fabrication techniques have allowed biomaterials scientists to fabricate surfaces
with architectures similar to a cell’s native environment. Examples of such fabrication methods
include phase inversion foaming procedures, particle leaching, and machining/etching
techniques.²⁻⁵ When researchers expose cells to these bio-inspired surfaces they are often
rewarded with enhanced cellular responses which include higher adhesion rates, higher
proliferation rates, more natural cellular morphologies, and better maintenance of phenotype.²⁻⁴,⁶

Chief among the fabrication methods used to generate biomimetic scaffolds is electrospinning.
Electrospinning is an old technique dating from the 1930s.⁷ However, it has achieved a revival in
recent years due to its ability to create nano- and micro-scaled fiber constructs very similar in
architecture to the native extracellular matrix. Subsequently, numerous biostable and
biodegradable synthetic polymers have been electrospun⁸⁻¹¹ in addition to many naturally
occurring biopolymers.¹²⁻¹⁵ Standard electrospinning produces fibers deposited in a random
fashion, but multiple methods have been developed to generate structures with aligned fiber
architectures.¹⁶⁻¹⁸

In Chapter 2 the synthesis of a novel methacrylic terpolymer produced by free radical
copolymerization of hexyl-methacrylate (HMA), methyl methacrylate (MMA), and methacrylic acid
(MAA) was described and the materials cytocompatibility was demonstrated.¹⁹ In this Chapter,
we test the hypothesis that electrospinning can be used to create highly relevant terpolymer cell
scaffolds with a variety of topographies through control of the glass transition temperature of the polymer and the electrospinning conditions. Furthermore, we expect these different topographies to control the number and morphology of adherent endothelial cells.

3.3. Materials and methods

**Synthesis of methacrylic terpolymer.** The synthesis of the methacrylic terpolymer was achieved through free radical polymerization as described in Chapter 2.

**Preparation of terpolymer fiber scaffolds.** Terpolymer fiber samples were produced by electrospinning. The terpolymer was dissolved in an equimolar mixture of ether and acetone to a final concentration of 13 % (g/mL). The solution was passed through a needle at a rate 16 ml/hr using a syringe pump. The collector was placed at a distance of 16 cm from the needle. A voltage of 23 kV was applied between the needle and the collector. The polymer solution was drawn into fibers by the applied voltage and deposited on the collector in the form of non-woven terpolymer mats.

Aligned fiber scaffolds were produced by electrospinning onto a rotating mandrel. All electrospinning parameters were the same as for random fibers with the exception of the distance between the needle and collector which was increased to 30 cm. The velocity of the rotating surface was controlled to vary the degree of fiber alignment. Fiber scaffolds were placed under vacuum overnight to remove residual solvent.²⁰

**Density measurements/void fraction calculations.** Terpolymer films were produced through solution casting 30 mL of a 10 % (g/mL) polymer solution from THF into 100 mL Teflon Petri dishes. The dishes were covered in aluminum foil to dry slowly overnight. Samples where were further dried over night in an oven at 55 – 60 °C and then in a vacuum oven over night at 55 – 60
°C to remove residual solvent. The density of the films and electrospun mats were measured for a variety of terpolymer compositions. A 12 mm biopsy punch was used to cut a disc from the sample of interest. The thickness of the sample was measured using vernier calipers. Care was taken to firmly hold the electrospun samples without compressing the sample. The volume of the sample was then calculated, and the sample was weighed using an analytical balance. Experiments were performed in triplicate and repeated three times for a total of 9 measurements per polymer composition. Void fraction ($\Phi$) was calculated using the following equation:

$$\Phi = \left(1 - \frac{\rho_{\text{electrospun}}}{\rho_{\text{film}}}\right) \times 100\% \text{ where } \rho \text{ is density.}$$

**Molecular weight analysis.** Gel permeation chromatography was performed as described in Chapter 2.

**Analysis of scaffold morphology.** The architecture of the oriented and random fiber scaffolds was examined using scanning electron microscopy (Philips XL30 ESEM FEG). Samples were sputter coated with gold and imaged using a 10 kV accelerating voltage. Fiber diameter, pore area, and fiber alignment of the terpolymer meshes were measured from the scanning electron micrographs by random counting of 60 fiber diameter, pore areas, and fiber angles using Image J image analysis software (Image J, Bethesda, MD). Experiments were repeated on samples electrospun from 3 distinct batches of material at each composition level so that data includes batch-to-batch variations in polymer processing.

**Preparation of cell culture surfaces.** Cell adhesion and growth experiments were performed in tissue-culture treated 24-well plates (BD Falcon, Franklin Lakes, NJ). The cell growth surfaces were first coated with a film of the desired polymer through solution casting of a 10 % (g/mL) solution of the desired polymer in THF. The electrospun meshes of the appropriate composition were cut to size using a biopsy punch. Since some of the scaffolds are highly porous, the film of polymer ensures that if cells are capable of penetrating the scaffold they will interact with a film of
the polymer instead of the growth surface of the well plate. Wells containing only solution cast films of polymer were used as negative controls while un-modified wells of tissue culture polystyrene (TCPS) were used as the positive control. Well plates were sterilized by exposure to UV radiation for 2 hours.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were maintained as described in Chapter 2.

**Cell adhesion and growth.** 1 mL of media containing 25,000 cells was added to each well of the well plate (12,500 cells/cm²) and incubated for the desired length of time. Unattached cells were removed with 3 washes with pre-warmed Dubelcco’s phosphated buffer saline (DPBS). The test surfaces were then covered with 1 mL of fresh PBS. Adherent cells were lysed using 3 cycles of freezing and thawing followed by sonication. 0.25 mL of a 5ug/mL solution of Hoechst 33258 was added to each chamber and allowed to incubate for 30 minutes under ambient conditions. 0.25 mL of cell lyses from each chamber was then transferred to 3 wells of a 96 well plate. Adherent cell numbers were determined by comparing the fluorescent intensity of each well to a calibration curve generated from a known number of cells. Cell densities were calculated by dividing the cell number by the area of the test surface. Experiments were performed in quadruplicate and the fluorescence for each well was measured three times.

**Cell enzymatic activity.** As an assessment of cell viability the enzymatic activity of the adherent cells was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD). This is a colorimetric assay based on the cleavage of a tetrasolium salt (WST-8) by mitochondrial dehydrogenases in viable cells. Increased enzyme activity leads to an increase in the amount of formazan dye which is measured with a spectrophotometer.
Culture plates were prepared as described in section 2.8. After a 7 day-incubation period, the cell culture medium was aspirated and fresh medium was added along with CCK-8 reagent (10:1 ratio). Plates were then incubated for 3 hours at 37 °C. After incubation, 100 µl of solution from each well was transferred into 3 wells of a 96-well plate, where the absorbance was measured at a wavelength of 450 nm. Wells containing media and CCK-8 reagent without cells were used to adjust for background signal. The experiment was repeated in quadruplicate.

**Cell morphology.** Morphology was assessed as described in Chapter 2.

**Data analysis and statistical methods.** All averaged values are reported as average ± standard deviation. To determine statistical difference when comparing two means, a students’ t test was used. To determine statistical difference when comparing multiple means an ANOVA was first used to determine if any pair of means were statistically different and then a Tukey-Kramer Honestly Difference test was used to determine which pairs of means were statistically different (α < 0.05).

### 3.4. Results and discussion

#### 3.4.1 Fabrication and characterization of electrospun scaffolds

Much of the current research in the field of biomaterials involves creating materials which mimic *in vivo* tissue through the incorporation of mechanical, structural, chemical, and biological cues. In this Chapter, we discuss the fabrication of a biomimetic electrospun cell scaffold made from a novel methacrylic terpolymer and the effect that the resulting scaffold has on the number and morphology of adherent HUVECs.
The amount of HMA and MMA incorporated into the polymer molecules is specified through control of synthesis conditions. Thus materials with a wide range of physical properties can be generated allowing this polymer system to be used in a variety of biomedical applications.\textsuperscript{19} MAA is incorporated at a constant 2 mole % to introduce chemical functionality for possible post-synthesis derivatizations.\textsuperscript{21,22} For the remainder of this Chapter, the polymers are classified by the mole percent of HMA added to the polymerization reaction. For instance H20 refers to a material polymerized from an HMA/MMA/MAA molar composition of 20/78/2 and H90 refers to a material polymerized from a 90/8/2 molar composition. Various compositions of the methacrylic terpolymer have been fabricated into cell scaffolds through electrospinning. Figure 3.1 displays scanning electron micrographs of the resulting fiber constructs.
Figure 3.1. Scanning electron micrographs of terpolymer scaffolds of various chemical compositions: (A) H20 low magnification, (B) H20 high magnification, (C) H40 low magnification, (D) H40 high magnification, (E) H60 low magnification, (F) H60 high magnification, (G) H90 low magnification, (H) H90 high magnification.
The low magnification panels in Figure 3.1 (A, C, E, and G) illustrate that large areas of uninterrupted polymer fibers can be generated through electrospinning. Furthermore, these panels illustrate that this polymer system can be used to generate scaffolds with a wide variety of topographies. These range from very open structures displaying large amounts of porosity (panel A) to much more fused structures composed of much smaller levels of total porosity (panel G). Generated under the same electrospinning conditions, the differences in topography can be explained by examination of the glass transition temperature of the respective polymers. H20 (panel A) is rich in methyl methacrylate and thus is well below its glass transition temperature. During the spinning process, the material is electromechanically drawn into rigid fibers prior to deposition. When the ambient electrospinning conditions exceed a polymer’s glass transition temperature (H90, panel G) the polymer has a more pronounced viscous component to its physical behavior resulting in a more closed scaffold architecture displaying fused fibers. To ensure the topographical changes occurred due to the $T_g$ and not because of residual solvent in the fibers both solution concentration and flight distance from needle to collector were varied. In all circumstances the rubbery materials produced low porosity scaffolds indicating that the changes in topography are due to the $T_g$ of the material.

The higher magnification panels in Figure 3.1 (B, D, F, and H) also reveal the effects of $T_g$ on individual fiber surface topography. Again, large differences are observed as the base material transitions from a glassy (panel B) to a rubbery polymer (panel H). The rubbery state yields smooth fibers, while the glassy state produces fibers exhibiting considerable surface roughness. During the electrospinning process, the outside of the polymer solution jet likely dries first, creating a film of solidified polymer surrounding a core swollen with solvent. As the remaining solvent evaporates, the volume of the core shrinks and the outer skin crumples resulting in rough surface features. The materials near or above their glass transition temperature exhibit relaxation processes which markedly reduce this surface roughness produced during electrospinning. Scaffold architecture was explored in more detail through measurement of average fiber
diameter, average pore area, and average void fraction. The results from these measurements are compiled in Table 3.1 along with the modulus of the terpolymer films.\textsuperscript{19}

<table>
<thead>
<tr>
<th>Composition</th>
<th>$T_g$ (°C)</th>
<th>Film modulus (MPa)</th>
<th>Fiber diameter (µm)</th>
<th>Pore area (µm$^2$)</th>
<th>Void space (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H10</td>
<td>81 ± 6</td>
<td>504 ± 73</td>
<td>68 ± 64</td>
<td>8100 ± 10300</td>
<td>68 ± 7</td>
</tr>
<tr>
<td>H20</td>
<td>69 ± 5</td>
<td>438 ± 24</td>
<td>12 ± 12</td>
<td>2200 ± 1400</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>H30</td>
<td>59 ± 4</td>
<td>295 ± 46</td>
<td>8 ± 6</td>
<td>1100 ± 670</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>H40</td>
<td>50 ± 4</td>
<td>346 ± 39</td>
<td>6 ± 3</td>
<td>760 ± 490</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>H50</td>
<td>41 ± 2</td>
<td>214 ± 41</td>
<td>6 ± 3</td>
<td>900 ± 700</td>
<td>75 ± 8</td>
</tr>
<tr>
<td>H60</td>
<td>23 ± 2</td>
<td>74 ± 56</td>
<td>5 ± 2</td>
<td>690 ± 440</td>
<td>70 ± 10</td>
</tr>
<tr>
<td>H70</td>
<td>12 ± 2</td>
<td>18 ± 14</td>
<td>5 ± 1</td>
<td>440 ± 260</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>H80</td>
<td>3 ± 4</td>
<td>15 ± 5</td>
<td>6 ± 4</td>
<td>230 ± 240</td>
<td>22 ± 8</td>
</tr>
<tr>
<td>H90</td>
<td>-6 ± 2</td>
<td>3 ± 2</td>
<td>6 ± 3</td>
<td>270 ± 190</td>
<td>18 ± 8</td>
</tr>
</tbody>
</table>

Table 3.1. Polymer glass transition temperature, film modulus, and characterization of random electrospun fiber scaffolds.

All polymer compositions explored in this study were capable of being electrospun into fiber constructs with average fiber diameters in the micron range. More glassy polymers produced fibers with larger fiber diameters and wider standard deviations, while the fibers quickly became narrower and more uniform in size as the material entered the rubbery state. Corresponding with the change in fiber diameters, the pore area and the void fraction of the scaffolds also decreased with decreasing MMA concentration. The pore area (the area of the polygons produced by the upper most layers of fibers) drops from 2200 µm$^2$ for H20 to 270 µm$^2$ for H90, almost an order of magnitude decrease. The void fraction (a measurement of the amount of open space in the scaffold) decreased from a maximum of 84 % void space in H20 to 18 % void space in H90. Interestingly, the pore area and void fraction do not vary linearly with polymer composition. The scaffolds display a sharp decrease in void fraction and pore area between H60 and H70.
indicating that the material must be above its glass transition temperature before the viscous component of the material is prominent enough to largely affect the scaffold architecture.

In addition to being able to create scaffolds with randomly deposited fibers, aligned fiber constructs were generated by depositing fibers onto a rotating surface.\textsuperscript{16-18} Controlling the rotation speed of the collector allows control of the degree of fiber orientation within the scaffold. Scanning electron micrographs of aligned fiber constructs are shown in Figure 3.2.
For the fiber orientation study, the H20 polymer system was chosen since it produces the most discrete and easily viewed fibers. Visually it can be seen in Figure 3.2 that fiber alignment is enhanced as the rotation rate of the collector surface is increased from 1.8 meters per second to 15.2 meters per second. The degree of fiber alignment was characterized by measuring the angle of fiber deviation from the direction of rotation. The histograms in Figure 3.3 illustrate that fibers are increasingly deposited in the direction of rotation as the rotation rate increases. The partially aligned fiber mats were characterized further by measuring average fiber diameter, average fiber deviation from the direction of rotation, and average pore area. The results from this study are shown in Table 3.2.
Figure 3.3. Distribution of fiber orientation for H20 fibers collected onto surfaces with different rotational speeds: (A) 1/8 m/s, (B) 5.5 m/s, (C) 9.9 m/s, and (D) 15.2 m/s.
<table>
<thead>
<tr>
<th>Collector speed</th>
<th>Fiber diameter</th>
<th>Fiber orientation</th>
<th>Pore area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>avg</td>
<td>min</td>
<td>max</td>
</tr>
<tr>
<td><strong>Rotation rate</strong></td>
<td><strong>(RPM)</strong></td>
<td><strong>Tangential speed</strong></td>
<td><strong>avg</strong></td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>12 ± 6.8</td>
<td>2.7</td>
</tr>
<tr>
<td>60</td>
<td>1.8</td>
<td>12 ± 5.1</td>
<td>6.0</td>
</tr>
<tr>
<td>180</td>
<td>5.5</td>
<td>10 ± 4.0</td>
<td>5.2</td>
</tr>
<tr>
<td>325</td>
<td>9.9</td>
<td>9.9 ± 3.6</td>
<td>5.1</td>
</tr>
<tr>
<td>500</td>
<td>15.2</td>
<td>8.8 ± 2.5</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Table 3.2. Average fiber diameter, pore area, and fiber orientation of H20 fibers collected onto surfaces with different rotation speeds.
The average diameter of the fibers decreases as the rotation rate of the collector increases likely due to a tangential force applied to the fibers during deposition which acts to draw the fibers into alignment. Similar results have been previously reported.\textsuperscript{23-26} The average angle of fiber deviation is 45 $\pm$ 29 degrees when the collector is stationary corresponding to a fully random deposition pattern, but the average deviation angle decreases to 6 $\pm$ 7 degrees when the collector is rotated at 15.2 m/s. As the fibers become more aligned, they pack closer together eliminating pore space. The pore area of the materials collected at 9.9 and 15.2 m/s could not be measured through the method described above. Multiple sets of aligned surfaces were generated, all with similar results to what is shown above, illustrating reproducibility in the alignment process.

The physical performance of polymer materials is highly tied to the average molecular weights of the polymer system.\textsuperscript{27} Therefore, we wished to determine if the electrospinning process was degrading the polymer chains. Four batches of material were selected, each at a different composition level, and the number average molecular weight of unprocessed and electrospun samples were measured using gel permeation chromatography (GPC). The results of this study are shown below in Figure 3.4. A t-test was used to determine if the average molecular weight of the electrospun material was significantly less than that of the unprocessed material ($\alpha < 0.5$). From this study, we conclude that the electrospinning processes does not significantly decrease the number average molecular weight of the polymer, regardless of the composition of the polymer chain.
3.4.2. Cellular response to electrospun scaffolds

HUVECs were used as model cells in this research. By varying the ratio of HMA to MMA in the polymer we can tune the physical properties of the biomaterial. For instance, the Young’s Modulus of the polymer films is presented in Table 3.1 and ranges from 3 – 500 MPa as we previously reported. Though modulus of the substrate can affect the behavior of adherent cells in our previous work no statistical difference was seen in the behavior of HUVECs seeded onto films of H20, H60, and H90. All surfaces were able to foster a confluent monolayer of spread and highly viable HUVECs after 2 weeks of \textit{in vitro} culture illustrating the cytocompatibility of the material.\textsuperscript{19} Since the HUVECs are relatively insensitive to substrate stiffness this polymer system
allows a facile method of screening how different electrospun scaffold architectures affect the biological response of HUVECs. In this research, HUVECs were seeded onto random fiber scaffolds of H20, H60, and H90 and aligned fiber scaffolds of H20 and the adherent cell number, enzymatic activity, and morphology of the HUVECs were assessed after four different incubation conditions: 4 hours in serum un-enriched media, 4 hours in serum enriched media, 4 days, and 7 days. Films of H90 were used as the negative control and TCPS was used as the positive control.

The results from the cell adhesion study are presented in Figure 4.5. Focusing on the 4 hour adhesion results, we see that the presence of serum increases the number of attached cells, most likely due to the adsorption of ligand-containing proteins from solution onto the biomaterial surface which promote cellular attachment. After 4 hours with serum, there is no statistical difference in the adhesion of cells to the electrospun scaffolds. However, after 4 and 7 days we see that the H90 random fiber scaffolds adhere a statistically greater number of cells than any of the other terpolymer scaffolds indicating that the HUVECs present on this scaffold display an increased rate of proliferation. After 7 days the H90 random fiber scaffold fostered approximately twice as many adherent cells in comparison to the negative control. The cell adhesion results are corroborated by measurements of enzymatic activity after 7 days (Figure 3.6). We see increased enzymatic activity of cells adherent to the H90 random fiber scaffolds in comparison to all other terpolymer biomaterials. In fact, HUVECs adherent to the H90 random fiber scaffold displayed a 5 fold increase in enzymatic activity in comparison to the negative control.
Figure 3.5. HUVEC adhesion and growth onto electrospun terpolymer scaffolds. Letters above each column indicate statistical differences. Averages linked by the same letter are not statistically different.
Figure 3.6. Metabolic activity of HUVECs adherent to electrospun terpolymer scaffolds after 7 days. Letters above each column indicate statistical differences. Averages linked by the same letter are not statistically different.

The morphology of the adherent cells was also evaluated after 7 days of incubation. The results from this study are presented in Figure 3.7. As observed with the fluorescence images, the scaffold topography drastically affects the morphology of the adherent cells. We can see that HUVECs on the H90 films (panel B) and the H60 fiber scaffolds (panel D) have begun the spreading process. However, they do not display the degree of spreading as seen on the TCPS positive control (panel A). DAPI-stained nuclei on the H20 random fiber scaffolds (panel E) were located on multiple focal planes indicating that the open structure enabled the cells to penetrate into the scaffold. Furthermore, the actin cytoskeletons also spanned multiple focal planes indicates that the cells obtaining a more three dimensional morphology. The most interesting morphological data comes from the H90 random fiber scaffolds (panel C) and the H20 aligned
fiber scaffolds (panel F). In addition to creating a surface which increases cellular proliferation, the H90 random fiber scaffold also resulted in an enhancement of cell spreading. In fact, the fluorescence microscopy images illustrate that cells on this scaffold are very similar in morphology in comparison to the HUVECs on the positive control. Though increased cellular proliferation was not observed on the H20 aligned fiber scaffolds, we do see that cells are preferentially elongated in the direction of fiber orientation as described in other studies.  

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28-30
Figure 3.7. Fluorescence microscopy images of HUVECs seeded on scaffolds of various surface topographies for 7 days at 20x magnification: (A) tissue culture polystyrene, (B) H90 film, (C) H90 random fiber scaffold, (D) H60 random fiber scaffold, (E) H20 random fiber scaffold, and (F) H20 aligned fiber scaffold.
The results from this study make sense from a biological perspective. In native vasculature, endothelial cells are present on the luminal surface in a confluent monolayer and attached to an underlying basal lamina.  These cells have a spread morphology and are elongated in the direction of blood flow due to the shear stress of the passing fluid.  Though vasculature is a fibrous structure composed primarily of elastin and collagen, these protein fibers often have nanometer fiber diameters.  In this study the HUVECs are interacting with fibrous structures with micron fiber diameters.  HUVECs interacting with the H60 and H20 random fiber scaffolds are interacting with a structure with fibers and pores on the same size scale as the cells.  These scaffolds present the cells with the wrong structural cues and as a result the cells showed hindered spreading and proliferation and low enzymatic activity.  In fact, the large gaps between fibers may have acted to isolate individual cells from one another, preventing cell-cell interactions, and hindering cellular processes. The H90 random fiber scaffold presented cells with a relatively flat surface allowing for firm attachment, growth, and spreading in a two dimensional monolayer, yet also provided increased surface area and topography which allowed the cells to more easily attach and spread in comparison to a flat film.  As a result, the cells exhibited more robust proliferation and increased cellular spreading.  Though the cells attaching to the H20 aligned fiber scaffold did not display increased proliferation, the fluorescence images illustrate that the alignment of fibers can coax the cells to obtain an elongated morphology parallel to that of the direction of fiber alignment, more similar to native morphology.  Though the more porous H60 and H20 random fibers scaffolds were not appropriate for HUVECs, these scaffolds may be better for the adhesion and growth for cells present in three dimensional structures in vivo.

In Chapter 2, HUVECs showed similar adhesion and growth characteristics on the methacrylic terpolymers regardless of material stiffness indicating that the increased HUVEC proliferation and enhancement of spreading observed in this work is largely due to the incorporation of the appropriate topography into the scaffold.  However, the terpolymer scaffolds are still not able to
promote the same growth as the TCPS positive control after 7 days of culture. Chapter 4 describes the covalent attachment of peptide ligands to the terpolymer in order to further enhance the cell adhesion characteristics of the polymer.

3.5. Conclusions

A family of methacrylic terpolymers were electrospun into fibrous cell scaffolds. The scaffold morphology varied with the glass transition temperature of the polymer: glassy material resulted in very porous scaffolds with discrete fibers while rubbery materials resulted in much more closed structures with fused fibers. This polymer system provided a method for screening how scaffold architecture affected the adhesion, growth, and morphology of HUVECs. After 4 hours of incubation with serum enriched media none of the electrospun scaffolds adhered a statistically larger population of cells. However, after 4 and 7 days of culture we see that the H90 random fiber scaffolds foster a statistically larger population of HUVECs in comparison to a polymer film and the other electrospun scaffolds. These results are corroborated by enzymatic activity data which shows an increase in metabolic activity of HUVECs on these scaffolds. Through fluorescence microscopy we see that the HUVECs attached to the H90 fibrous scaffolds have a much greater degree of cell spreading, similar to what is seen on the TCPS positive control. HUVECs attached to the H20 aligned fiber scaffold have preferentially elongated morphology paralleling the direction of scaffold fiber alignment. These data illustrate that behavior of HUVECs can be modified through appropriate biomaterial design and fabrication.
Chapter 4: Selective Endothelial Cell Attachment to Peptide Modified Biomaterials

4.1. Abstract

Using phage display technology, 12-mer peptide ligands were discovered which bind with high specificity to human blood outgrowth endothelial cells (HBOCs). Methacrylic terpolymer biomaterials containing these ligands were synthesized along with materials containing the RGD and RGE tripeptide units. Chain transfer chemistry, a novel method of biofunctionalization, was used to incorporate the ligands into the biomaterial. In static culture experiments in serum free media increased HBOC attachment was observed on materials containing one of the novel 12-mer peptides while up regulated HUVEC attachment was only observed on the RGD-containing materials. These results illustrate the utility of one of the novel HBOC ligands to enhance cell attachment to a synthetic biomaterial and also illustrate the specificity of the novel ligands towards HBOCs. The enhanced cellular attachment was only observed in serum free media conditions.

4.2. Introduction

Though endothelial progenitor cells (EPCs) and their outgrowths possess many characteristics which make them useful as a therapeutic tool, the cell populations are still poorly characterized. This means that many of the surface receptors presented by the cells are unknown.\textsuperscript{1-4} Previously we have designed a novel methacrylic terpolymer system, and in this chapter we wish to covalently incorporate peptide ligands which specifically interact with receptors present on the
membranes of late outgrowth EPCs – also known as the human blood outgrowth endothelial cell (HBOECs).\textsuperscript{1,5,6} Furthermore, it is desirable for these ligands to be specific to HBOECs and not other commonly occurring blood cell types (including mature endothelial cells).

To discover such peptides, our collaborator Dr. Anka Veleva at North Carolina State University used phage display technology. The technique of phage display was first reported in 1985 by Dr. George Smith in \textit{Science}.\textsuperscript{7} At the heart of this technology is the filamentous bacteriophage (or phage), a virus particle which is only capable of infecting certain gram-negative bacterial cells such as \textit{E. coli}. The phage has relatively simple construction, a single DNA strand surrounded by a protein capsule. The DNA strand codes for the proteins which make up the capsule, while in turn the protein shell protects the genetic material. Commonly, the M13 strain of the Ff bacteriophage is used in display experiments.\textsuperscript{8} An artistic illustration of the M13 virus is shown in Figure 4.1.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{m13_virus.png}
\caption{Artistic illustration of the M13 bacteriophage protein capsule.\textsuperscript{3}}
\end{figure}

P IX, p VII, p VIII, p VI, and p III represent proteins which comprise the bacteriophage capsule. The DNA (containing 11 genes) is stored in the cylindrical portion of the capsule, while the p III proteins are active in binding to receptors in bacterial cell membranes such as the pili located on \textit{E. coli} surfaces.
In phage display, virus particles are transformed through the introduction of new genetic material. Most often this additional genetic information is introduced to gene 3 of the phage which results in the expression of an additional amino acid sequence displayed at the terminal end of the p III proteins. In his original publication, Smith illustrated that the transformation of the phage particles does not inhibit their ability to infect bacteria or their ability to replicate. Large libraries of transformed phage particles can be generated, each displaying a unique amino acid sequence. Figure 4.2 provides an artistic illustration of a subset of a phage library.

![Artistic illustration of a subset of a phage display library. Each shape at the terminus of the p III protein indicates a unique amino acid sequence being displayed to the external environment.](image)

Figure 4.2. Artistic illustration of a subset of a phage display library. Each shape at the terminus of the p III protein indicates a unique amino acid sequence being displayed to the external environment.

Once the library of phage particles as been generated, it is then incubated with the target and particles displaying appropriate polypeptide units (ligands) bind with the target. Non-adherent
phage particles (the one which did not specifically interact through receptor-ligand interactions) are removed through centrifugation and washing. This step (commonly referred to as biopanning) is repeated multiple times to ensure selection of the tightest binding ligands.

The displayed ligand (phenotype) is directly related to the transformed DNA of the phage particle (genotype). After the binding experiments, the phages of interest are amplified through infection of *E. coli*, creating enough genetic material to perform gene sequencing and thus determine the exact amino acid sequence which showed binding affinity toward the target.\textsuperscript{9} Since its development phage display has become a powerful tool for biological, clinical, and biotechnological applications such as characterization of receptor- and antibody-binding sites, the study of protein-ligand interactions, and drug discovery.\textsuperscript{10-13}

In this chapter we describe the use of phage display to discover novel peptides which bind with high specificity to HBOECs but not other commonly occurring blood cell types. We hypothesize that by covalently incorporating these peptides into our methacrylic terpolymer system will result in polymer interfaces which promote the specific attachment of HBOECs and not other commonly occurring cells found in circulation.

### 4.3. Materials and Methods

**Peptide synthesis.** All peptide sequences were synthesized using standard Fmoc chemistry on a solid phase peptide synthesizer (AI Biotech, Inc., Richmond, VA). The peptides were purified by high performance liquid chromatography (HPLC) and chemical purity was confirmed by MALDI-TOF mass spectrometry.

**Synthesis of peptide-modified polymers.** The synthesis of the methacrylic terpolymer was achieved through free radical polymerization as described in detail in Chapter 2. The desired
amount of peptide was also added to the polymerization reaction to allow peptide incorporation through chain transfer chemistry.

**Determination of peptide concentration in polymer.** Amino acid analysis was performed by Al Biotech, Inc. to determine the concentration of peptide in the polymer materials. The amino acid sequences in a known mass of polymer were hydrolyzed in 1mL of an equivolume propionic acid:hydrochloric acid solution for 2.5 hours at 150°C. The samples were vortexed 4x during hydrolysis to ensure dispersion of the amino acids into the hydrolysis solution. HPLC was used to determine the moles of amino acids. Peptide density of the materials were determined by dividing the number of moles of amino acids by the mass of the polymer sample.

**Molecular weight.** Gel permeation chromatography was used as described in Chapter 2.

**HBOEC isolation and culture.** The use of human materials described in this study was approved by the responsible ethical committee. Buffy coat mononuclear cells were obtained from 100 mL fresh peripheral blood by density gradient centrifugation using Histopaque 1077 (Sigma, St. Louis, MO). Buffy coat mononuclear cells were re-suspended in endothelial growth media (EGM-2) (Cambrex Bioscience, Walkersville, MD) without further subpopulation enrichment and placed into one well of a six well plate coated with type I collagen (BD Biosciences, Bedford, MA) and placed in an incubator. Non-adherent cells were removed after 48 hours and every second day thereafter. Colonies with cobblestone morphology appeared after 3 weeks in culture. The cells were grown until larger colonies were formed. Colonies were selected, trypsinized, and expanded over several passages.

**HUVEC culture.** Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained as described in Chapter 2.
Preparation of cell culture surfaces. Peptide-grafted materials were dissolved in acetone and dip-coated on glass coverslips (18-mm, Fisher Scientific, Pittsburg, PA). Vacuum-dried samples were sterilized by immersion in 70% ethanol. After washing with PBS, the coverslips were placed in tissue culture polystyrene plates and blocked with 1% BSA in PBS at 37°C for 1 hour. Surfaces were rinsed with PBS.

Endothelial cell adhesion. Test surfaces were incubated with a HBOEC or HUVEC suspension in serum-free or serum-containing medium at a concentration 20,000 cells/mL. After 2 hours of incubation, the medium was aspirated and loosely attached cells were washed off with three changes of PBS. Adherent cell density was measured using the Dojindo Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) according to manufacturer instructions. Briefly, attached cells were re-fed with EGM-2 containing 10% CCK-8 reagent (Dojindo Molecular Technologies, Gaithersburg, MD) for spectrophotometric determination of relative cell binding. EGM-2 containing 10% CCK-8 was added to three wells without cells to serve as a background control. After incubation at 37°C for 3 hours, 100 µl of solution from each well was transferred into a separate 96-well plate, where the absorbance of the samples against a background control was measured at a wavelength of 450 nm on a Wallac-VICTOR™ microplate reader (Perkin - Elmer, Wellesley, MA). Cell binding was normalized to attachment of cells to a surface modified with negative control peptide.

Data analysis. Data are representative of at least three independent experiments and quantitative analyses are presented as means ± SD. Statistical analysis, where applicable, was performed in Microsoft Excel. A two-tailed unpaired Student’s t-test was used to compare the differences. A value of α < 0.05 was considered statistically significant.
4.4. Results and Discussion

The library of phage particles were prescreened against HUVECs to remove peptide sequences which show affinity for mature endothelial cells. The remaining particles in the phage library were incubated with HBOECs for three rounds of biopanning. The remaining phages were amplified through *E. coli* infection, and binding peptides were determined through DNA sequencing.\textsuperscript{14} From this work two potential peptide ligands were selected for further analysis and a GGGSC spacer group was added to the C-terminus of both sequences. The primary amino acid sequences of these polypeptides are shown in Table 4.1 along with the sequence of RGD and RGE sequences used in this work. In solution assays both phage display peptides showed tight and high affinity binding to HBOECs but not HUVECs, human lymphocytes, or human neutrophils (data not shown).

<table>
<thead>
<tr>
<th>Primary Amino Acid Sequence</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPSLEQRTVYAKGGGSC</td>
<td>TPS</td>
</tr>
<tr>
<td>SYQTLKQHLPYGGGGGSC</td>
<td>SYQ</td>
</tr>
<tr>
<td>CGRGDSY</td>
<td>RGD</td>
</tr>
<tr>
<td>CGRGESY</td>
<td>RGE</td>
</tr>
</tbody>
</table>

Table 4.1. Primary amino acid sequence of polypeptide ligands used in this research.

To incorporate the peptides into the terpolymer biomaterial chain transfer chemistry was used. This reaction, which occurs between the free radical of a growing polymer chain and a thiol group (S-H), has long been used in the polymer industry as a method of controlling polymer molecular weight and in this work we use it as novel approach to incorporating bioactive species into a material.\textsuperscript{15-17} The use of chain transfer to functionalize the materials is possible due to the cysteine residue that terminates each sequence. A schematic of chain transfer chemistry is
illustrated in Figure 4.3. Materials containing all four peptide sequences shown in Table 4.1 were produced.

```
A  Active-Peptide-GGGSC-S-H
   ↓
B  Active-Peptide-GGGSC-S*
   ↓
C  Active-Peptide-GGGSC-S
```

Figure 4.3. Schematic of chain transfer chemistry: (A) the thiol group of a polypeptide ligand encounters a growing polymer chain, (B) chain transfer occurs which results in the termination of the growing polymer chain and the transfer of the radical (*) to the polypeptide, and (C) the free radical continues adding monomer producing a polymer chain which is terminated with the polypeptide.

Two techniques were used to confirm the covalent attachment of the ligands to the polymer chains: amino acid analysis and gel permeation chromatography. Amino acid analysis was used to measure the moles of peptide in a given mass of polymer. However, one could envision free peptide being trapped in the polymer matrix. To illustrate that chain transfer was occurring GPC was employed. Chain transfer reactions result in a decrease in molecular weight of the polymer chains so batches of polymer in which chain transfer occurred should have lower number average molecular weights. The results from these studies are shown in Table 4.2 and as expected the amino acid analysis confirmed the presence of the ligand in the material and we see the expected drop in number average molecular weight indicating that the peptide was covalently incorporated through the chain transfer method.
<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>Peptide Concentration</th>
<th>$M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(--)</td>
<td>(µmol peptide/g polymer)</td>
<td>(kDa)</td>
</tr>
<tr>
<td>Terpolymer</td>
<td>N/A</td>
<td>223</td>
</tr>
<tr>
<td>TPS-terpolymer</td>
<td>2.26</td>
<td>168</td>
</tr>
<tr>
<td>SYQ-terpolymer</td>
<td>2.30</td>
<td>108</td>
</tr>
<tr>
<td>RGD-terpolymer</td>
<td>1.90</td>
<td>218</td>
</tr>
<tr>
<td>RGE-terpolymer</td>
<td>2.26</td>
<td>210</td>
</tr>
</tbody>
</table>

**Table 4.2.** Peptide concentration within biomaterials and number average molecular weight.

To assess the bioactivity of these materials HBOEC and HUVEC adhesion studies were performed in both serum-free and serum-containing medium. The results from these studies are presented in Figure 4.4. Fibronectin (FN) coated glass is used as the positive control in these studies and results are normalized to the negative control (RGE-containing materials).
Focusing on the results from the serum-free experiments, we see increased cellular attachment of HUVECs was obtained on the RGD-containing materials and increased cellular attachment of HBOECs was obtained on the TPS-containing materials (Figure 4.4, Panels A and B). These data indicate that chain transfer chemistry can be used to incorporate polypeptide ligands into a polymer biomaterial while maintaining the ligand’s bioactivity. Furthermore, the specificity of HUVECs to the RGD-modified materials and the HBOECs to the TPS-modified materials illustrates that these two cell populations are phenotypically different and that the amino acid sequences found through phage display target a surface marker unique to HBOECs in comparison to HUVECs. Increased cellular attachment was not observed on materials containing the SYQ peptide. Two possible reasons for this are 1) the ligand loses bioactivity when
conjugated to a solid surface or 2) not enough ligand was present in the material to result in an increase in cell attachment. Future work will include increasing the ligand density in the materials to see if a larger amount of peptide results in a stronger cellular response.

Though the results in serum-free media were encouraging, no difference was observed in the cell adhesion to any of the biomaterial surfaces when serum was present in the media (Figure 4.4, Panels C and D). We believe this is due to the adsorption of proteins to the biomaterial interface which acts to hide the peptide ligands from view of the cells.¹⁸

4.5. Conclusions

Polypeptide ligands were discovered which bind with high specificity to human blood outgrowth endothelial cells (HBOECs) but not other common cell in circulation (including mature endothelial cells). These ligands were then covalently incorporated into a polymeric biomaterial through the novel method of chain transfer chemistry. The incorporation of the ligands was confirmed through amino acid analysis and gel permeation chromatography. During cell adhesion tests, HUVECs showed increased attachment to only RGD-containing materials while HBOECs showed increased attachment to only TPS-containing materials. These results illustrate that chain transfer chemistry can be used to incorporate peptide ligands into a biomaterial without the loss of ligand bioactivity. These results illustrate the phenotypic difference between surface markers expressed on HUVEC and HBOEC surfaces. Furthermore, we have used these phenotypic differences to engineer interfaces tuned to the selective adhesion of certain endothelial cell populations.
Chapter 5: PEGylated Terpolymer Biomaterials with Non-Fouling Interfacial Properties

5.1. Abstract

A terpolymer copolymerized from hexyl methacrylate (HMA), methyl methacrylate (MMA), and poly(ethylene glycol) methacrylate (PEGMA) was synthesized. Polymers containing 0 to 25 mole % PEGMA were studied. As the mole fraction of PEGMA in the polymer chains increased, the material becomes more hydrophilic as observed by a decrease in the contact angle of water (81° to 68°) and an increase in the equilibrium water absorption (0.7 to 237 wt %). Furthermore, the material shows non-fouling interfacial properties through resistance to protein adsorption and cellular attachment. 1.2 μg/cm² fibrinogen, 18,000 HUVECs/cm², and 3,000,000 platelets/cm² adsorbed or adhered on non-PEGylated materials while very low amounts of protein or cells were observed on materials containing ≥ 15 mole % PEGMA. Being a thermoplastic, the polymer can be processed post-synthesis. To illustrate the processing capabilities of the material, polymer solutions were electrospun into non-woven fibrous scaffold which also retained their non-fouling character.

5.2. Introduction

Implanted biomaterials often trigger the body’s defensive and reconstructive processes which can undermine device function. Often these undesired biological processes are mediated through interactions at the biomaterial interface with cells and macromolecules present in the host environment. For example, implants can initiate the foreign body response resulting in the
isolation of the implant through the development of a fibrous capsule and a state of chronic inflammation both of which can lead to poor healing and impaired performance of the device.\textsuperscript{1-3} As described in Chapter 1, thrombus is of chief concern for blood-contacting biomaterials.\textsuperscript{4-7}

In order to improve the function of biomedical devices and promote better healing, researchers have generated non-fouling surfaces which minimize these undesired interactions with the biological environment.\textsuperscript{1} Both natural and synthetic molecules have been discovered which when present at the interface make the surface non-fouling. Such chemical motifs include poly(ethylene glycol); poly(hydroxyethyl methacrylate); zwitterions such as phosphorylcholine, sulfobetaine, and carboxybetaine; and polysaccharides such as hyaluronic and alginic acids.\textsuperscript{8-14} Many thermodynamic factors play a role in determining the fouling character of interfaces; however, when designing non-fouling interfaces two characteristics seem necessary: (1) hydrophilicity and (2) electrical neutrality. These two characteristics result in surfaces which hold their waters of hydration and thus resist the deposition of proteins and the adhesion of cells.\textsuperscript{1,15}

In previous Chapters, we have described the design, synthesis, and characterization of a family of bioactive methacrylic terpolymers intended for use in cardiovascular devices.\textsuperscript{16-21} In addition to the possibility of initiating thrombosis \textit{in vivo} previous experiments have illustrated that adsorption of serum proteins onto the biomaterials surface negated the activity of covalently incorporated peptide ligands.\textsuperscript{22,23} Therefore, we wish to create materials which resist protein adsorption and non-specific cellular attachment.

In this Chapter we tested the hypothesis that incorporation of poly(ethylene glycol) (PEG) chemical moieties would impart non-fouling interfacial properties to our polymer system. The methacrylic acid was removed from these formulations and replaced with various mole fractions of poly(ethylene glycol) methacrylate (PEGMA).
5.3. Materials and Methods

**Polymer synthesis.** The terpolymer was produced through free radical polymerization as described in Chapter 2. The monomers used in this reaction were n-hexyl methacrylate (HMA) (Alfa Aesar, Ward Hill, MA), methyl methacrylate (MMA) (ACROS Organics, Pittsburgh, PA), and poly(ethylene glycol) methacrylate (PEGMA) (poly(ethylene glycol) (400) monomethyl ether monomethacrylate, Poly Sciences, Warrington, PA). Upon termination of the reaction polymers synthesized from 0 – 15 mole % PEGMA were extracted in excess 1:1 methanol:deionized water to remove unreacted species. Materials synthesized from more than 15 mole % PEGMA were soluble in the methanol:deionized water mixture, so were extracted in pure deionized water. The extracted polymer was collected, dried, and weighed.

**Composition analysis.** The composition of the terpolymer was determined through $^1$H Fourier transform nuclear magnetic resonance (NMR) spectroscopy. 600 μL of a 0.03 g/mL polymer solution in deuterated chloroform was transferred to a Wilmad NMR sample tube (Warminster, PA). The NMR analysis was performed on a DMX 600 MHz NMR machine at 300 K. 128 scans were used to construct the NMR spectra. A peak at 4.0 ppm represents the two protons adjacent to the oxygen atom in the HMA pendant group, a peak at 3.7 ppm represents the three protons present in the terminal methyl group of the MMA pendant group, and a peak at 3.5 ppm represents the three protons present in the terminal methyl group of the PEGMA pendant group. Additional peaks arose from protons in the PEGMA repeat unit and overlapped the MMA peak. To correct for this extra signal pure poly(PEGMA) was analyzed through NMR and it was found that for each 1 unit of area present in the 3.5 ppm peak, 10.5 units of area were present in the range from 3.55 – 3.8 ppm. The molar ratio of HMA, MMA, and PEGMA in the polymer was determined by taking the integral of the peak at 4.1 ppm and dividing it by 2 for the two protons it
represents; the integral from 3.55 – 3.8 ppm, subtracting off the extra signal due to PEGMA protons and dividing the remaining signal by 3 for the three protons it represents; and taking the integral of the peak at 3.5 and dividing it by 3 for the three protons it represents. The sum of the measurements was normalized to 100 in order to determine the mole percent of each repeat unit present in the polymer material.

**Molecular weight analysis.** Polymer molecular weight was analyzed through gel permeation chromatography (GPC) as described in Chapter 2.

**Water absorption.** Water absorption studies were performed as described in Chapter 2.

**Static contact angle.** A 2 μL droplet of deionized water was pipetted onto a thin terpolymer film and the shape of the drop was captured using a camera. The contact angle was measured as the angle through the denser phase using ImageJ analysis software (Image J, Bethesda, MD).

**Fibrinogen adsorption.** For all experiments involving fibrinogen and HUVEC adhesion polymer of a given composition was selected from one of the polymerization batches. The culture surface of a 96 well plate was coated with the polymer of interest through the addition of 35 μL of a 10% polymer solution (g/mL) in THF. The well plate was covered in aluminum foil and dried slowly over night followed by drying in an oven and vacuum oven for 24 hours each at 55 – 60°C. The wells were equilibrated in PBS over night at 37°C. The PBS was aspirated and 50 μL of a 50 μg/mL solution of fluorescently tagged fibrinogen (Alexa Fluor 488 conjugated fibrinogen, Invitrogen, Carlsbad, CA) was added to each well. The protein solution was allowed to interact with the surface for 40 minutes at 37°C before the solution was aspirated and the wells were washed 5x with PBS to removed reversibly adsorbed fibrinogen. 50 μL of PBS was added to each well and the fluorescence intensity was measured by a plate reader. Eight wells per polymer composition were read and the fluorescence of each well was measured three times.
Fluorescence intensity was converted to adsorbed protein mass through comparison to a calibration curve generated from known amounts of tagged protein.

**Preparation of film surfaces for cellular adhesion and growth studies.** The growth surface of a 24-well plate was coated with 150 μL of a 10% solution of the desired polymer in THF, covered with aluminum foil and allowed to dry slowly for 24 hours at ambient conditions to prevent bubbles, then dried in an oven at 55 – 60 °C for 24 hours to remove residual solvent.

**Preparation of fibrous surfaces for HUVEC adhesion and growth studies.** Terpolymer fiber samples were produced by electrospinning as described in Chapter 3.

**HUVEC culture.** Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained as described in Chapter 2.

**Adhesion and growth of HUVECs.** Well plates were sterilized through exposure to UV radiation for 2 hours and equilibrated overnight in PBS prior to the introduction of cells. HUVEC adhesion and growth experiments were carried out as described in Chapter 3.

**Morphology of HUVECs.** HUVEC morphology was analyzed as described in Chapter 2.

**Preparation of platelet adhesion surfaces.** Pre-cleaned 12 mm glass coverslips were dip coated in a 10% polymer solution in THF. Coverslips were dried in a convection oven at 55 – 60 °C for 24 hours and then dried at 55 – 60 °C in a vacuum oven to remove residual solvent. Coverslips were then transferred to the wells of a 24 well plate.

**Collection of platelets.** Blood was collected from a healthy and aspirin-abstaining 27 year old male through venipuncture into blood collection tubes containing a 3.2% sodium citrate solution
(9:1 final dilution of blood to sodium citrate). Blood samples were centrifuged at 300g for 15 minutes to isolate the platelet rich plasma (PRP). The PRP was collected and transferred to a conical tube. The blood was further centrifuged at 2000g for 15 minutes to isolate the platelet poor plasma (PPP). The PPP was collected and added to the PRP to create the final platelet suspension. The platelet concentration was determined through use of a hemocytometer and found to be 189,000 platelets/μL. Platelets were used immediately.

**Platelet adhesion and morphology experiments.** The well plates were sterilized through exposure to UV radiation for 2 hours, and equilibrated in PBS overnight. 300 μL of platelet suspension was pipetted onto each of the test surfaces (28.4 million platelets/cm²) and allowed to interact for 1 hour in an incubator. Cultures were washed 3 times with DPBS to remove non-adherent platelets. Cultures were then fixed with a 4% solution of paraformaldehyde for 2 hour. Cultures were rinsed 3 times with deionized water to remove residual salt, dried, sputter coated, and analyzed through scanning electron microscopy (Philips XL30 ESEM FEG) using a 10 kV accelerating voltage. Images were taken of 5 randomly selected fields of view on each test surface. The number of adherent platelets were counted and divided by the area of the image in order to calculate the adherent cell density. Three test surfaces of each polymer composition were used and TCPS was used as the positive control. The morphology of the platelets was analyzed through observation of the SEM images.

**Statistical analysis.** When more than two means were compared, analysis of variance (ANOVA) was used to determine if any pairs of means were statistically different. If the ANOVA indicated statistical difference then the Tukey-Kramer Honestly Difference Test was used to determine which of the pairs of means were statistically different. In all tests α < 0.05.
5.4. Results and Discussion

5.4.1. Polymer synthesis and characterization

A schematic of the HMA/MMA/PEGMA copolymer structure is shown in Figure 5.1. In this manuscript the polymer is referred to by the mole percent of PEGMA used in the synthesis. For instance, P15 refers to a material copolymerized from 20/65/15 mole % HMA/MMA/PEGMA. The PEGylated materials are compared to the base polymer (P0) synthesized from 20/80 mole % HMA/MMA.

![Polymer structure schematic](image)

Figure 5.1. Schematic of polymer chemical structure.

Regardless of the molar ratio of monomer used in the synthesis reaction, the yield from the polymerization reaction was approximately 50%. NMR and GPC analysis were used to
determine the composition and molecular weight averages of the polymers. Sample NMR spectra are shown in Figure 2 of an (A) HMA/MMA copolymer, and (B) a PEGMA homopolymer, and (C) an HMA/MMA/PEGMA terpolymer. As evident from Figure 5.2, overlapping occurred between peaks in the two spectra. However, as described in the Materials and Methods section the peak signals were decoupled allowing for analysis of the terpolymer composition. Results from the polymer composition and molecular weight studies are compiled in Table 5.1. As these data show, there is a slight decrease in the mole percent of MMA in the polymer in comparison to the feed as reported in a previous study. However, the terpolymer composition tracks closely with the feed composition illustrating that polymer with a wide range of compositions can be synthesized. Furthermore, the standard deviations of the composition data are small indicating reproducibility in the synthesis process. The number average molecular weight of the terpolymer decreased from 270 KDa for P0 to 90 KDa for P25. For all the synthesis reactions the mass of monomer used was held constant at 20 g. Since the molecular weight of the PEGMA monomer is much greater than the molecular weight of the other comonomers this resulted in the decrease of the number of moles of monomer present in the reactor. This most likely led to a decrease in the rate of polymerization and thus the production of shorter polymers chains.
Figure 5.2. $^1$HNMR spectra: (A) 20/80 mol % HMA/MMA copolymer, (B) PEGMA homopolymer, and (C) 20/78/2 mol % HMA/MMA/PEGMA terpolymer.
Table 5.1. Polymerization conditions, polymer composition, and molecular weight averages for PEGylated terpolymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed composition (mol % HMA/MMA/PEGMA)</th>
<th>Polymer composition (mol % HMA/MMA/PEGMA)</th>
<th>Mn (KDa)</th>
<th>Mw (KDa)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>20/80/0</td>
<td>22/78/0 ± 0/0/0</td>
<td>272 ± 15</td>
<td>556 ± 6</td>
<td>2.04 ± 0.14</td>
</tr>
<tr>
<td>P5</td>
<td>20/75/5</td>
<td>22/72/6 ± 0/1/1</td>
<td>231 ± 21</td>
<td>424 ± 34</td>
<td>1.84 ± 0.06</td>
</tr>
<tr>
<td>P15</td>
<td>20/65/15</td>
<td>22/63/15 ± 1/1/1</td>
<td>140 ± 19</td>
<td>217 ± 30</td>
<td>1.55 ± 0.06</td>
</tr>
<tr>
<td>P25</td>
<td>20/55/25</td>
<td>20/56/25 ± 1/2/1</td>
<td>87 ± 5</td>
<td>138 ± 5</td>
<td>1.55 ± 0.06</td>
</tr>
</tbody>
</table>

Polymer/water interactions were probed through swelling and static contact angle experiments.

From swelling experiments, all polymer samples reached equilibrium water uptake after approximately 2 days. The equilibrium water absorption values are compiled in Table 5.2. From these data we can see that materials with various swelling properties can be synthesized, from a hydrophobic material which adsorbs less than 1 wt% water to very hydrophilic materials which adsorb greater than 230% water. Materials containing > 35 mole % PEGMA were found to be water soluble. As the mole fraction of PEGMA in the polymer increased the surface hydrophilicity also increased as observed by a decrease in the contact angle of water. The results from the static contact angle experiments are compiled in Table 5.2 where we see the average contact angle for the P0 polymer is 81 degrees and this value drops to 68 degrees for the P25 material. The average contact angles for the P0 and P5 materials are statistically greater than the P15 and P25 materials. Though increasing concentrations of PEGMA did result in a decrease in contact angle, one would have expected the decrease to be more dramatic. However, this mild increase in wetability can be rationalized through the amphipathic nature of the polymer. The materials rich in PEGMA are very rubbery and have a lot of molecular motion. During the film casting process one could envision the hydrophilic PEG pendant groups retreating from the hydrophobic air environment resulting in a relatively hydrophobic interface. Thus during static contact angle analysis all surfaces would have similar hydrophilicity. However, when exposed to an aqueous...
environment for prolonged times the hydrophilic pendant groups could migrate to the surface resulting in greater surface wetability and the desired non-fouling character.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Equilibrium water absorption (weight %)</th>
<th>Average contact angle (degrees)</th>
<th>Maximum (degrees)</th>
<th>Minimum (degrees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>0.7 ± 0.6</td>
<td>81 ± 4</td>
<td>93</td>
<td>75</td>
</tr>
<tr>
<td>P5</td>
<td>8 ± 3</td>
<td>78 ± 5</td>
<td>88</td>
<td>69</td>
</tr>
<tr>
<td>P15</td>
<td>60 ± 5</td>
<td>67 ± 6*</td>
<td>74</td>
<td>53</td>
</tr>
<tr>
<td>P25</td>
<td>237 ± 5</td>
<td>68 ± 9*</td>
<td>74</td>
<td>43</td>
</tr>
</tbody>
</table>

Table 5.2. Interactions of PEGylated terpolymer with water. Static contact angle averages marked with asterisks (*) are statistically less than unmarked averages.

5.4.2. Biological interactions

The HMA/MMA/MAA termpolymer formulations we studied previously adsorb proteins readily and are adhesive to many varieties of cells. These non-specific interactions with biological systems could lead to undesired consequences such as thrombosis in the presence of blood. Therefore, PEGMA monomer was copolymerized into the terpolymer in order to create a material with non-fouling biological properties. To probe the non-fouling capabilities of the terpolymer protein adsorption, HUVEC adhesion, and platelet adhesion studies were performed. In all biological experiments the P0 material is used as the negative control to illustrate how PEGylation alters the biological properties of the material in comparison to previously studied formulations.

Since we are interested in developing this polymer system for blood contacting biomaterial applications, fibrinogen – a blood protein which plays a key role in the development of thrombus –
was selected as our model protein. Figure 5.3 compiles the results from the fibrinogen adsorption experiments. TCPS and P0 adsorb 0.8 – 1.2 µg of fibrinogen/cm² which corresponds to essentially a full monolayer of protein coverage. The P0 surface adsorbed a statistically larger amount of protein, possibly due to its hydrophobic nature which has been shown to enhance biofouling due to the entropic benefits of liberating the hydrophobically bound waters of hydration during the sorption event. P5 adsorbed less than half of the fibrinogen of the positive control TCPS while P15 and P25 adsorbed very small amounts of protein. It was found that protein adsorption to the P15 and P25 materials were not statistically different indicating that further resistance to protein fouling does not occur for formulations synthesized from greater than 15 mole % PEGMA.

![Graph showing fibrinogen adsorption to PEGylated terpolymers.](image)

Figure 5.3. Fibrinogen adsorption to PEGylated terpolymers. Pairs of means not linked by the same letter are statistically different.
Figure 5.4 summarizes the adhesion and growth of HUVECs on the PEG-terpolymer films. As expected, we see adhesion and growth of the cells on the TCPS and P0 surfaces over the 15 days of incubation. However, as the mole percent of PEG in the material increases, we see the surfaces becoming increasingly difficult for the cells to inhabit. After 5 and 15 days of incubation, we see that the P5 surface does have a population of adherent cells; however, statistically fewer cells are capable of attachment compared to the TCPS and P0 surfaces. The P15 surface is even more efficient at resisting the attachment and growth of the HUVECs resulting in a surface which is essentially cell free after 15 days of adhesion.

Figure 5.4. Adhesion and growth of HUVECs on PEGylated terpolymers. Pairs of means not linked by the same letter are statistically different.
The results shown in Figure 5.4 for HUVEC adhesion and growth are supported by the fluorescence microscopy images shown in Figure 5.5. After 5 days of incubation, we see a large population of endothelial cells on the surface of the P0 biomaterial. However, as the concentration of PEG increases in the material, we see that fewer cells capable of inhabiting the polymer surface resulting in P15 and P25 surfaces which are nearly cell free. From the fluorescence images we can also see that the presence of the PEG also inhibits cell spreading since individual cells on the P5 and P15 surfaces cover less surface area than the cells on the P0 surface.
Along with fibrinogen, platelets are the other key player in thrombus development. Figure 5.6 shows SEM images of terpolymer surfaces exposed to a suspension of human platelets in blood plasma. From the images, we see a sharp decrease in platelet adhesion between P5 and P15. The SEM images were quantified, and the results are shown in Figure 5.7. We can see that the base polymer material (P0) is statistically less adhesive towards platelets than the positive control of TCPS. Also, we see that incorporation of 5 percent of the PEGMA monomer does not reduce the adhesion of platelets. However, P15 and P25 adhered statistically fewer cells than the P0 and P5 surfaces. In fact, almost no platelets were found on these surfaces. Furthermore, the
platelets on the P0 and P5 surfaces are spread and agglomerated indicating they have been activated into a pro-thrombogenic state.¹

Figure 5.6. Scanning electron micrographs of platelets adherent to PEGylated terpolymers at 600x magnification: (A) P0, (B) P5, (C) P15, (D) P25.
Figure 5.7. Adhesion of platelets to PEGylated terpolymers. Pairs of means not linked by the same letter are statistically different.

Though poly(ethylene glycol) is a common polymer for use in hydrogel biomaterials, this material is often limited to thermosetting applications since the polymer is water soluble when not crosslinked.\textsuperscript{27-29} By copolymerizing the polar PEGMA with the relatively hydrophobic HMA and MMA we were able to produce thermoplastic non-fouling materials. Many studies have been produced recently which probe cellular interactions with biomaterials fabricated into structures similar in architecture to the extracellular matrix (ECM).\textsuperscript{30-32} In this study, electrospinning was employed to produce non-woven scaffolds of PEGylated terpolymer to demonstrate that non-fouling fibrous scaffolds can be generated. Figure 5.8 shows SEM images of the electrospun terpolymer scaffolds. As expected, the glassy P0 polymer produced a scaffold with high porosity and discrete fibers; however, as the materials becomes more rubbery the scaffold obtains a more closed morphology with fused fibers as described in Chapter 3. Since the materials rich in PEG
are very hydrophilic, we feared the fibers would swell in the presence of water resulting in occlusion of the pore structure. To image the scaffolds in their swollen state, environmental SEM was employed. From Figure 5.8 panel E we can see that the morphology of the P0 scaffold is largely unaffected by the presence of water which is reasonable considering the polymer only absorbs ~1% of water by weight. Panel F shows that the very hydrophilic P25 scaffold (which swells >200% by weight in the presence of water) also retains its porous nature.
Figure 5.8. Scanning electron micrographs of electrospun PEGylated terpolymers at 250x magnification: (A) P0 dry, (B) P5 dry, (C) P15 dry, (D) P25 dry, (E) P0 swollen, (F) P25 swollen.

In Chapter 3 we illustrated that endothelial cells favorably interact with fibrous scaffolds similar in morphology to the P15 and P25 scaffolds as illustrated by increased proliferation, spreading, and enzymatic activity. However, we expect the presence of the PEGMA to undermine the adhesion of proteins, platelets and HUVECs regardless of the favorable topography. Figure 5.9 shows the adhesion of HUVECs to PEG-terpolymer fibers after 4 days of culture. From the
figure, we can observe that increasing amounts of PEG results in decreased cellular attachment. However, the P15 fibrous scaffolds adhered a statistically greater number of cells than the P15 films indicating the topography incorporated through electrospinning did result in the scaffold holding an increased number of cells. However, it is possible that these cells are physically trapped in the polymer fiber matrix instead of being biologically attached to the scaffold through receptor mediated interactions. In the future we intend to incorporate cell binding peptides in the PEO containing systems to see if specific HUVEC and HBOEC adhesion can be enhanced in these materials.

![Figure 5.9. HUVEC adhesion to electrospun PEGylated terpolymers scaffolds after 4 days of incubation. Pairs of means not linked by the same letter are statistically different.](image-url)
5.5. Conclusion

Polymeric biomaterials were synthesized through the copolymerization of hexyl methacrylate, methyl methacrylate, and poly(ethylene glycol) methacrylate. Incorporation of the PEGMA repeat unit resulted in increased polymer hydrophilicity and non-fouling character. Polymers containing $\geq 15$ mol % PEGMA were found to have excellent resistance to fibrinogen adsorption and platelet and HUVEC adhesion. Furthermore, copolymerizing the hydrophilic PEGMA with the relatively hydrophobic HMA and MMA resulted in the synthesis of a water insoluble thermoplastic material with non-fouling interfacial properties which was processed into fibrous scaffolds through electrospinning. These fiber structures retained their non-fouling capacity as illustrated through resistance to HUVEC adhesion.
6.1. Abstract

A methacrylic terpolymer with non-fouling interfacial properties was synthesized through the random copolymerization of hexyl methacrylate, methyl methacrylate, and sulfobetaine methacrylate (a monomer baring a zwitterionic pendant group). Polymers were synthesized from feeds containing 0 – 30 mol % of the zwitterionic methacrylate. Proton NMR quantified the incorporation of sulfobetaine methacrylate into the polymer structure. Water absorption studies and static contact angle experiments illustrated the increasing hydrophilicity of the material with increasing zwitterion concentration. The biological properties of the polymer were probed through fibrinogen adsorption, human umbilical vein endothelial cell adhesion and growth, and platelet adhesion. Strong resistance to protein adsorption and cell and platelet attachment was observed on the materials. Results were compared to the non-fouling behavior of the P15 terpolymer formulation described in Chapter 5. It was observed that the PEG-containing materials were slightly more effective at resisting HUVEC adhesion and growth over a 7 day incubation period, but the zwitterion-containing materials were equally effective at resisting fibrinogen adsorption and platelet adhesion.
6.2. Introduction

Although PEGylation is a powerful and commonly used technique for imparting non-fouling interfacial properties to a biomaterial, this technology does have limitations. Most notably, poly(ethylene glycol) suffers from oxidative degradation in the presence of oxygen or transition metal ions such as Cu(II) and Fe(III).\(^1\)-\(^4\) Since most biological environments contain such oxidizers one could envision a PEGylated interface eventually losing its non-fouling nature over time.

PEG’s susceptibility to degradation clearly illustrates the need to develop biostable surface modification techniques which result in non-fouling interfaces. One such technique is the functionalization of an interface with zwitterionic groups.\(^5\)-\(^7\) The cell membrane is composed of a phospholipid bilayer. The hydrophilic head group of the lipid molecules – which is displayed to the external environment - is a phosphorylcholine moiety.\(^8\) Since the cell membrane is a naturally non-fouling interface, researchers have attached lipids to biomaterials in order to resist biofouling.\(^9\)-\(^13\) Subsequent research illustrated that self assembled monolayers terminated in phosphorylcholine groups also resulted in non-fouling interfaces indicating the zwitterionic group was responsible for imparting the desired properties to the interface.\(^14\),\(^15\) A methacrylate molecule which bares such a phosphorylcholine pendant group was developed by Ishihara, and copolymers containing the phosphoryl choline monomer or surface grafted phosphoryl choline methacrylate brushes have been shown to create interfaces resistant to non-specific biological interaction.\(^16\)-\(^21\)

Although phosphoryl choline containing polymers are effective at generating non-fouling interfaces, the monomer is difficult to synthesize and purify.\(^22\) Therefore, new zwitterion-containing methacrylate monomers were developed, specifically carboxybetaine methacrylate
and sulfobetaine methacrylate. Furthermore, both monomers were utilized to create non-fouling interfaces.\textsuperscript{23-28}

In this Chapter, we wish to explore other avenues of imparting non-fouling character to our biomaterial system. Therefore, we wish to test the hypothesis that a sulfobetaine-containing terpolymer will have non-fouling properties competitive with the PEG-containing formulations as probed through protein adsorption, HUVEC adhesion and growth, and platelet adhesion.

6.3. Materials and Methods

**Monomer synthesis and structure verification.** Sulfobetaine methacrylate (SBMA) was synthesized through the ring opening reaction of propane sultone (Sigma-Aldrich, Milwaukee, WI) with dimethylamino ethyl methacrylate (Sigma-Aldrich). Each reactant was dissolved in acetone to a final concentration of 0.2 M and allowed to react for 2 days at ambient conditions. The SBMA precipitated out of the reaction solution as a white powder which was collected through filtration. The filter cake was washed 3x with acetone to remove unreacted species. The product was dried under vacuum and stored at 2°C. Monomer composition was determined through \textsuperscript{1}H Fourier transform nuclear magnetic resonance (NMR) spectroscopy in deuterated water. The NMR analysis was performed on a DMX 600 MHz NMR machine at 300 K. 128 scans were used to construct the NMR spectra.

**Polymer synthesis.** The terpolymer was produced through free radical polymerization in 50 mL of solvent using 10 grams of the desired monomer mixture as described in Chapter 2. The specific solvent, monomer ratio, initiator mass, and chain transfer agent volume are presented in Table 6.1.
Polymer composition analysis. The composition of the terpolymer was determined through $^1$H Fourier transform nuclear magnetic resonance (NMR) spectroscopy. 600 $\mu$L of a 0.03 g/mL polymer solution in deuterated chloroform was transferred to a Wilmad NMR sample tube (Warminster, PA). The NMR analysis was performed on a DMX 600 MHz NMR machine at room temperature. 128 scans were used to construct the NMR spectra. A peak at 4.0 ppm represents the two protons adjacent to the oxygen atom in the HMA pendant group, a peak at 3.7 ppm represents the three protons present in the terminal methyl group of the MMA pendant group, and a peak at 3.4 ppm represents the six protons present in the two methyl groups attached to the quaternary amine in the SBMA pendant group. Additional peaks arose from protons in the SBMA repeat unit and overlapped the HMA peak. It was found that for each 1 unit of area present in the 3.4 ppm peak, 0.67 units of area were present in the range from 4.2 – 3.9 ppm. The molar ratio of HMA, MMA, and PEGMA in the polymer was determined by taking the integral of the peak at 4.1 ppm and dividing it by 2 for the two protons it represents; the integral from 4.2 – 3.9 ppm, subtracting off the extra signal due to SBMA protons and dividing the remaining signal by 3 for the three protons it represents; and taking the integral of the peak at 3.4 and dividing it by 6 for the six protons it represents. The sum of the measurements was normalized to 100 in order to determine the mole percent of each repeat unit present in the polymer material.

Water absorption. Water absorption experiments were performed as described in Chapter 2.

Static contact angle. Contact angle experiments were performed as described in Chapter 5.

Fibrinogen adsorption. Protein adsorption experiments were performed as described in Chapter 5.

Preparation of film surfaces for cellular adhesion and growth studies. Polymer of the desired composition was solution cast onto the growth surface of two well glass chamber slide
from a 10% (g/mL) solution. The chamber slides were allowed to dry slowly under ambient conditions to minimize bubbles. Chambers slides were then dried in a convection oven and vacuum oven at 55 – 60°C each for 24 hours. Prior to use, chamber slides were sterilized by exposure to UV light for 30 minutes, 1.5 mL of sterile PBS was added to each well, and the chamber slides were incubated over night at 37°C.

**HUVEC adhesion, growth and morphology experiments.** HUVECs were cultured and maintained as described in Chapter 2. HUVEC adhesion, growth, and morphology experiments were performed as described in Chapter 5.

**Cytotoxicity assay.** 1.5 mL of medium containing 40,000 HUVECs was added to each well (10,000 cells/cm²) and incubated for 3 hours. After the incubation period, the medium along with the unattached cells were collected and transferred to Permanox wells and allowed to incubate for 3 additional hours and 24 hours. At each time point cell behavior was analyzed through phase contrast microscopy at 10x magnification.

**Platelet experiments.** Platelet isolation, adhesion experiments, and analysis were performed as described in Chapter 5.

**Statistical analysis.** When more than two means were compared, analysis of variance (ANOVA) was used to determine if any pairs of means were statistically different. If the ANOVA indicated statistical difference then the Tukey-Kramer Honestly Difference Test was used to determine which of the pairs of means were statistically different. In all tests $\alpha < 0.05$. 

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6.4. Results and Discussion

6.4.1. Polymer synthesis and characterization

The sulfobetaine methacrylate monomer was synthesized through the reaction of dimethylamino ethyl methacrylate and propane sultone as seen in Figure 6.1A. The chemical composition of the monomer was verified through $^1$H NMR. A sample spectrum of the zwitterionic monomer is shown in Figure 6.1B. The unlabeled peak arises from small amounts of non-deuterated solvent (water).
Figure 6.1. (A) Reaction of dimethylamino ethyl methacrylate with propane sultone to form sulfobetaine methacrylate, and (B) the $^1$H NMR spectrum of the sulfobetaine methacrylate monomer.

The monomers were copolymerized in order to create polymer structures as seen in Figure 6.2A. The molar concentration of zwitterion used in polymer synthesis ranged from 0 to 30 mol % Table 6.1 contains the polymerization conditions used to generate the biomaterials. Material polymerized from $\leq 5$ mol % of SBMA were polymerized in DMF; however, materials polymerized from $\geq 15$ mol % SBMA were polymerized in methanol else the materials precipitated prematurely.
during polymerization. Also, greater amounts of initiator and dodecanethiol (a chain transfer agent) were used for material polymerized from ≥ 15 mol % SBMA in order to decrease the average molecular weight of the polymer chains else materials were intractable after synthesis. Regardless of the synthesis conditions, all reactions resulted in an approximately 50% yield. The properties of the zwitterionic polymer are compared with the P15 polymer developed in Chapter 5 (Figure 6.2B).
Figure 6.2. Schematic of (A) sulfobetaine containing terpolymer and (B) poly(ethylene glycol)-containing terpolymer.
Table 6.2: Polymer synthesis conditions. Monomer ratios marked by an asterisk (*) contain PEGMA instead of SBMA.

The polymer composition was characterized through $^1$H NMR analysis. The resulting polymer compositions are reported in Table 6.2. The composition of the Z0, Z5, and P15 polymers are very similar to the initial charge of the chemical reactor. However, we see an accumulation of the zwitterionic co-monomer in the Z15 and Z30 formulations. This increase is likely due to reactivity ratios, a concept in copolymerization theory which describes the propensity of a radical species to add to a monomer of the same species as itself versus a different species. The accumulation of SBMA in the polymer could be due to its preferential addition compared to HMA or MMA. GPC experiments were attempted on these materials; however, the SBMA-containing polymers were not soluble in the appropriate solvents (tetrahydrofuran or toluene) even with the addition of LiCl.$^{29}$
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Polymer Composition (mol % HMA/MMA/SBMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z0</td>
<td>72/22/0 + 3/3/0</td>
</tr>
<tr>
<td>Z5</td>
<td>77/19/4 + 1/1/0</td>
</tr>
<tr>
<td>Z15</td>
<td>64/17/19 + 4/2/2</td>
</tr>
<tr>
<td>Z30</td>
<td>62/0/38 + 4/0/4</td>
</tr>
<tr>
<td>P15</td>
<td>22/63/15 + 1/1/1 *</td>
</tr>
</tbody>
</table>

Table 6.2: Polymer molecular characterization. Polymer compositions marked by an asterisk (*) contain PEGMA instead of SBMA.

The interactions of the polymer with water were measured through equilibrium water absorption experiments and static contact angle experiments. The results from these studies are shown in Table 6.3. From these data we can see that materials with various swelling properties can be synthesized, from a hydrophobic material which adsorbs approximately 1 wt% water to a more hydrophilic material which adsorbs approximately 50 wt%. As SBMA concentration increases the surface hydrophilicity also increases as observed by a decrease in the contact angle of water. The Z0 materials have an average contact angle of 90 degrees and this value drops to 66 degrees for the Z30 material. Interestingly materials polymerized from 5 and 15 mol % SBMA do not show a decrease in their water contact angle. This experimenter expected the decrease in water contact angle to be more dramatic; however, this mild increase in wetability can again be rationalized through the amphipathic nature of the polymer as described in Chapter 5.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Equilibrium Water Absorption</th>
<th>Static Contact Angle</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z_0)</td>
<td>(%)</td>
<td>(°)</td>
<td>(°)</td>
<td>(°)</td>
</tr>
<tr>
<td>Z0</td>
<td>1.02 ± 0.37</td>
<td>90 ± 5</td>
<td>82</td>
<td>100</td>
</tr>
<tr>
<td>Z5</td>
<td>24 ± 10</td>
<td>88 ± 9</td>
<td>77</td>
<td>103</td>
</tr>
<tr>
<td>Z15</td>
<td>49 ± 5</td>
<td>87 ± 11</td>
<td>71</td>
<td>100</td>
</tr>
<tr>
<td>Z30</td>
<td>42 ± 10</td>
<td>66 ± 11 *</td>
<td>51</td>
<td>85</td>
</tr>
<tr>
<td>P15</td>
<td>60 ± 5</td>
<td>67 ± 6 *</td>
<td>43</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 6.3: Biomaterial interactions with water. Static contact angle data marked with an asterisk (*) is statistically less than unmarked values.

6.4.2. Biological interactions

Since we are interested in developing these materials for blood contacting biomedical device applications, the biological properties of this family of materials was probed through interactions with blood components: Fibrinogen – the blood protein which crosslinks to form fibrin clots – was used as our model protein in protein adsorption assays; HUVECs were used to assess the adhesion and growth capacity of adherent cells to these materials; and platelets were used to assess the thrombogenic nature of the surface.

The results from the fibrinogen adsorption assay are seen in Figure 6.3. From these data we can see that the TCPS positive control and the Z0 biomaterial adsorb comparable quantities of fibrinogen (essentially a full monolayer of coverage). Materials polymerized from only 5 mol % of zwitterionic monomer absorbed half the protein as the positive control while Z15 materials show excellent resistance to protein adsorption and are comparable with the negative control (P15).
Figure 6.3. Fibrinogen adsorption to terpolymer biomaterials.

The non-fouling character of the terpolymer was also observed through the adhesion and growth of HUVECs and the adhesion of platelets. Figure 6.4 shows the growth characteristics of HUVECs on the biomaterial. From these data we see that the Permanox positive control, Z0, and Z5 are all capable of fostering a large endothelial cell population. Furthermore, on these surfaces, we see statistically larger levels of cellular attachment after 4 hours of incubation when serum is present in comparison to serum-free conditions. Though materials polymerized from 5 mol % sulfobetaine methacrylate do not show suppressed cellular attachment, statistically fewer HUVECs are observed on the Z15 materials. Though the Z15 interfaces are effective at resisting the adhesion and growth of HUVECs the negative control P15 surfaces adhered statistically fewer cells.
Figure 6.4. HUVEC adhesion and growth on terpolymer biomaterials.

The data in Figure 6.4 is supported by the fluorescence images of HUVECs on the terpolymer surfaces after 6 days of incubation as seen in Figure 6.5. From these data we see a near confluent monolayer of endothelial cells on the Permanox positive control and on the Z0 materials. The Z5 materials have a large number of adherent HUVECs; however, these cells are not highly spread indicating they may have increased difficulty inhabiting the Z5 interface. Both the Z15 and Z30 surfaces have large areas unoccupied by cells, similar to what is observed on the P15 negative control.
Figure 6.5. Fluorescence images of HUVECs adherent to zwitterionic terpolymer biomaterials after 6 days of incubation: (A) Permanox, (B) Z0, (C) Z5, (D) Z15, (E) Z30, (F) P15.
Surfaces can be functionalized with cationic chemical moieties to create biocidal interfaces. Though the surfaces studied in this research are electrically neutral, permanent charges do exist so we wished to probe the acute contact biocidal properties of these polymers so contact biocide experiments were performed. In these experiments HUVECs were incubated with the Z15 and Z30 surfaces for 3 hours. As observed in Figure 6.6 (Panels B and C) the cells were unable to adhere to the interface and instead formed clusters by forming cell-cell interactions. After the 3 hour incubation period the medium along with the unattached cells were transferred to Permanox wells to see if the cells were capable of regaining their original behavior as observed by adhesion capability and morphology. Three hours after replating we see the cell clumps have settled to the interface and have begun to interact with the cell culture substrate (Panels D and E). 24 hours after replating (Panels F and G) we can see that the cells have formed a monolayer on the tissue culture surface very similar to what is seen in cells seeded directly onto the Permanox (Panel A). These results illustrate that the majority of cells remained viable after interacting with the zwitterionic biomaterials for 3 hours. After the 24 hour incubation period, almost all of the cells which were incubated with the Z15 surface had regained natural function; however, many "floaters" were observed in wells containing the Z30-incubated cells. These data illustrate that a population of cells were unable to regain natural function indicating a biocidal effect of the Z30 materials towards HUVECs.
Figure 6.6. Phase contrast images of HUVECs: (A) 3 hours on Permanox, (B) 3 hours on Z15, (C) 3 hours on Z30, (D) Z15 cells after 3 hours of being replated on Permanox, (E) Z30 cells after 3 hours of being replated on Permanox, (F) Z15 cells after 24 hours of being replated on Permanox, and (G) Z30 cells after 24 hour of being replated on Permanox.
As an assessment of the blood compatibility of the polymer material platelet adhesion experiments were performed. Scanning electron micrographs of the biomaterial interfaces after exposure to platelets is shown in Figure 6.7. From these images we can see the Z0 terpolymer interface (panel A) fosters a large population of adherent platelets. Furthermore, they are agglomerated and spread indicating they have been activated into their pro-thrombogenic state. However, the Z5, Z15 and Z30 interfaces (panels B, C, and D) are largely platelet free illustrating the zwitterion-containing material’s ability to undermine the adhesion of platelets from blood plasma.

Figure 6.7. Scanning electron micrographs of platelets adhering to terpolymer biomaterials: (A) Z0, (B) Z5, (C) Z15, and (D) Z30.
The surface density of platelets on each biomaterial surface is quantified in Figure 6.8. In this Figure we see that the positive control (TCPS) adheres a very large population of platelets. The Z0 material adheres a statistically smaller population of cells in comparison to the positive control; however, all zwitterion-containing materials had very low adhesion of platelets. Furthermore, no statistical difference was observed between the zwitterionic materials and the P15 negative control indicating that either functional group is effective minimizing platelet attachment at a biomaterial interface.

![Figure 6.8. Quantification of platelet adhesion to terpolymer biomaterials.](image-url)
6.5. Conclusions

A family of sulfobetaine-containing methacrylic terpolymers were synthesized. The composition of the polymer was probed through $^1$H NMR. Due to the hydrophilic nature of the SBMA monomer the hydrophilicity of the polymer was expected to increase with increasing SBMA concentration as was verified through equilibrium water absorption and static contact angle experiments. The biological properties of the polymer were assessed through fibrinogen adsorption, HUVEC adhesion and growth, and platelet adhesion. The Z15 materials showed robust resistance to protein adsorption, similar to the P15 materials. The Z15 materials also showed a large decrease in the number of adherent HUVECs after 7 days of culture, though the P15 interfaces adhered a statistically smaller population of cells. However, the Z5, Z15, and Z30 materials all showed excellent resistance to the adhesion of platelets. The Z30 materials showed mild toxicity toward HUVECs; however, these data supports the Z5 and Z15 polymer systems for use as blood-contacting biomaterials.
Chapter 7: Conclusions and Future Work

7.1. Conclusions

The work presented in this dissertation describes the development of a novel methacrylic terpolymer biomaterials system for use in small diameter graft applications. By controlling the glass transition temperature of the polymer the physical behavior of the material can be tailored. For instance, materials with moduli ranging from 3 to 500 MPa were produced. By probing HUVEC adhesion and growth, viability, and morphology we illustrated the cytocompatibility of the material system and determined that terpolymer modulus does not largely affect the ability of HUVECs to inhabit the surface. However, the degree of cell spreading on the TCPS positive control was greater than on the terpolymer materials indicating that improvements can be made to the material interface to promote endothelialization.

The terpolymer system was fabricated into three dimensional fibrous scaffolds through electrospinning. All polymers produced scaffolds with fiber diameters in the micron range. However, the morphology of the scaffolds are influenced by the glass transition of the polymer. For instance, rubbery H90 materials produced scaffolds with fused fibers and 18% void space while glassy H20 materials produced scaffolds with discrete fibers and 84% void space. Aligned fiber scaffolds were produced by electrospinning onto a rotating collector and it was observed that the rotation rate of the collector influences the degree of fiber alignment.

HUVECs were seeded onto the fibrous scaffolds and increased cellular proliferation, enzymatic activity, and spreading were observed on the low porosity H90 random fiber scaffolds in
comparison to a film of the material and the other fibrous scaffolds. Though the topography did increase the desired cellular behavior, greater cell numbers were observed on TCPS after 7 days of culture. HUVECs grown on the aligned fiber scaffolds did not display enhanced proliferation or enzymatic activity; however, the HUVECs on the aligned fiber scaffolds obtained an elongated morphology in the direction of fiber orientation, a morphology more similar to what is seen in vivo.

Phage display was used to determine 12-mer peptides which bind with high specificity to HBOECs. Two of these peptides (abbreviated TPS and SYQ) were covalently incorporated into the H20 biomaterial via chain transfer chemistry. H20 materials functionalized with the RGD and RGE tripeptide unit were also synthesized. The RGE-containing materials acted as the negative control for cell adhesion experiments while fibronectin coated glass acted as the positive control. On the terpolymer biomaterials, increased HUVEC adhesion was only observed on the RGD-containing materials while increased HBOEC adhesion was only observed on the TPS-containing materials. These data indicate that HUVECs and HBOECs are phenotypically different cell populations which express unique surface markers. Furthermore, the differences in surface marker expression were employed to create materials designed for selective endothelial cell attachment. Interestingly, increased HBOEC adhesion was not observed on the SYQ-containing materials. Possibly this ligand loses activity when covalently attached to the polymer chains or there was not enough ligand in the material to result in enhanced cellular attachment. Though these results are encouraging, the increase in cellular attachment was only observed in serum-free medium conditions possibly due to the adsorption of serum protein to the interface which act to negate the presence of the covalently incorporated peptide ligands.

To resist the adsorption of serum proteins and the attachment of undesired cell types, two new material formulations were developed. In the first formulation the methacrylic acid co-monomer was removed and replaced by poly(ethylene glycol) methacrylate (PEGMA). Polymers were polymerized from feeds containing 0 to 25 mole percent of the co-monomer. As the mole percent
of the PEGMA increased the hydrophilicity of the material also increased as observed by greater water absorption and a lower water contact angle. The non-fouling character of the materials was probed through fibrinogen adsorption, platelet attachment, and HUVEC adhesion. It was found that materials synthesized from \( \geq 15 \) mole % PEGMA strongly resisted biofouling. Furthermore, no improvement to the material’s resistance to biofouling was observed in polymers produced from feeds containing \( \geq 15 \) mole % PEGMA.

A second formulation of non-fouling polymer was produced. In this work the PEGMA co-monomer was removed and replaced with the zwitterionic sulfobetaine methacrylate (SBMA). Materials polymerized from feeds containing 0 to 30 mole percent of SBMA were synthesized. Though HUVECs were better able to inhabit the surface of the SBMA-containing materials after seven days of culture, the Z15 materials were competitive with the negative control at resisting fibrinogen adsorption and the Z5, Z15, and Z30 materials were competitive with the negative control at resisting the adhesion of platelets.

7.2. Future Work

In this dissertation, several surface modifications were employed to tailor the behavior of adhering cells. In future work, these strategies should be employed in a single polymer system. For instance a logical next step in this work would be to produce a non-fouling polymer which is ligand-conjugated and has been electrospun into a three dimensional fibrous scaffold. Careful experimental planning would allow researchers to determine which surface characteristics are necessary to produce a confluent monolayer of either mature endothelial cells or human blood outgrowth endothelial cells.

In Chapter 4, peptide concentrations were reported as bulk concentrations. However, for cellular adhesion events, it is the surface density of ligand which is important. Furthermore, the cellular
response is expected to be dose dependent meaning the more ligand present at the surface the
greater number of cells which will adhere and the stronger their adhesion will be. However, we
also expect to see a saturation point after which more ligand at the surface will not result in an
enhanced cellular response. Employing a surface characterization technique such as X-ray
photoelectron spectroscopy (XPS) should enable the density of ligand at the surface to be
measured. Furthermore, materials with a variety of ligand concentrations should be prepared
which will enable elucidation of the optimum ligand density to maximize the adhesion of mature
endothelial cells or human blood outgrowth endothelial cells.

Creating an interface which fosters a confluent monolayer of endothelial cells is a noble goal.
However, in addition to the presence of the cells, cell function is also vitally important.
Endothelial cells not only prevent coagulation, but when activated (through vascular injury, for
instance) can act in a pro-thrombogenic manner. Therefore, coagulation assays should be
employed to determine that the endothelium which develops on the biomaterial surface acts to
inhibit thrombus formation.
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**Chapter 6**


