PEPTIDE LINKED POLYMERS FOR CARDIOVASCULAR APPLICATIONS

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By

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ABSTRACT

The primary objective of this work was to design and carry out feasibility studies for a biomaterial to capture circulating endothelial colony forming cells (ECFCs). Such a material has the potential to generate a confluent functioning endothelium in vivo. A methacrylic polymer system including peptides linked to pegylated pendant groups has been synthesized. Cellular responses of human umbilical vein endothelial cells (HUVECs) and cord blood/peripheral blood ECFCs on functionalized polymer surfaces were evaluated both under static and flow conditions.

Static experiments were first conducted. A non-fouling polymer was synthesized from hexyl methacrylate (HMA), methyl methacrylate (MMA), poly (ethylene glycol) methacrylate (PEGMA) and CGRGDS peptides. The peptide was incorporated into the polymer system either by a chain transfer reaction or by coupling to an acrylate-PEG-N-hydroxysuccinimide (NHS) comonomer. The PEG tethers polymerize at one end into the polymer backbone and present succinimide moities to covalently bind peptides at the distal end. The accessibility of peptides in aqueous solution was promoted and nonspecific protein adsorption was reduced by the PEG. HUVECs and cord blood ECFCs were cultured on these surfaces in short term adhesion and long term studies. A difference in number and morphology of endothelial cells was observed depending on the method of peptide incorporation. Both cell types adhered well to polymer films containing NHS coupled RGD peptide
after 2 hours even in the presence of albumin but significant cell detachment occurred after four days. Polymer solutions were then electro-spun into fibrous scaffolds. Both non-fouling and peptide binding characteristics were retained after processing.

A set of peptides from a phage display library has been found to bind specifically to peripheral blood ECFCs, which are also called adult human blood outgrowth endothelial cells (HBOECs), but not HUVECs. These peptide were also incorporated into the PEG containing acrylic polymers. Adult HBOEC adhesion was significantly enhanced by one of the phage display peptide GHMDMSPHAVID-GGGS. The adhesion to GHM-film was half that of RGD-film but specific to HBOECs even in 10% serum medium. The other cord-derived cells, cord blood ECFCs, like HUVECs, had high adhesion to the RGD-film but low adhesion to RGE-films.

This use of acrylic-PEG-NHS comonomer permitted peptide binding activity in the presence of serum proteins and significantly reduced the amount of peptide needed to observe the specific binding of cultured adult HBOECs. However the effect of peptide and PEG work against each other with respect to cell adhesion and proliferation. Appropriate peptides increase cell attachment significantly initially but the incorporation of PEG decreased cell adhesion and proliferation in long term culture. Optimization of polymer composition was conducted. Series of polymers with three different peptide incorporation densities and three different PEG content were synthesized. The amount of cells remaining on the surfaces was monitored at five different time points from 2 hours to 8 days. Polymer with highest peptide density and 7 mol% PEG content showed the best results in both cell initial attachment and long
term growth. 7 mol% incorporation of PEG suppressed protein deposition without interfering with the proliferation of cord blood ECFCs over 8 days.

Finally, the initial adhesion of ECs was studied under radial flow conditions. The use of a radial flow chamber allows the study of the effect of peptide density and shear stress on cell adhesion. HUVECs, cord blood ECFCs and peripheral blood ECFCs/HBOECs were studied. The surface of the flow cell device was either coated with polymer films or covered by synthetic fibers. Spin-coating was applied to produce smooth polymer films while fibrous scaffolds were generated by electro-spinning. The polymer was composed of HMA, MMA, PEGMA and CGRGDS peptide. A shear-rate-dependent increase of the attached cells with time was observed with all cell types. Significant cell adhesion was only observed under low shear conditions. The adhesion of ECs increased on RGD linked polymer surfaces compared to polymers without adhesive peptides. The number of attached cord blood ECFCs and HBOECs was significantly higher than that of HUVECs within the entire shear rate range and surfaces examined. The adhesion of endothelial progenitor cells suggests they are a promising source for seeding vascular grafts under physiological conditions.

It is hoped that information gained from this research will lead to the design of materials that can specifically bind to the circulating ECFCs in whole blood which can result in an endothelialized surface.
Dedication
This document is dedicated to my family and all my mentors, past and present.
ACKNOWLEDGMENTS

Five years ago, I was a completely new for chemical engineer. More than once I doubted whether or not I could finish my project and earn the Ph.D. I was lucky enough to receive much encouragement and support from individuals surrounding me. I especially wish to thank my advisor, Professor Stuart L. Cooper who was the one to introduce me into the field of biomaterials. He gave me a lot of opportunity to present work at professional meetings and to publish papers in journals. He was also very patient and helpful in correcting my English and providing guidance and resources to complete this work. I also wish to thank Professor Jianjun Guan who gave me valuable suggestions for my research and Professor Michael Paulaitis for the use of some of his laboratory equipments.

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# TABLE OF CONTENT

1 INTRODUCTION ..................................................................................................................................1  
1.1 Motivation and Objectives .........................................................................................................1  

2 LITERATURE REVIEW ....................................................................................................................8  
2.1 Biomaterial ..................................................................................................................................8  
   2.1.1 Introduction of Biomaterial ..............................................................................................8  
   2.1.2 Implant-Tissue Interactions ..........................................................................................10  
   2.1.3 Vascular Grafts ...............................................................................................................12  
   2.1.4 Problems with Current Vascular Graft ........................................................................14  
2.2 New Strategy to Tailor the Interface .........................................................................................16  
   2.2.1 Endothelial Progenitor Cells ..........................................................................................16  
   2.2.2 Peptides .......................................................................................................................19  
   2.2.3 Poly Ethylene Glycol ....................................................................................................22  
2.3 Methacrylate Polymers .............................................................................................................24  

3 MATERIALS AND METHODS ........................................................................................................27  
3.1 Polymer Synthesis .....................................................................................................................27  
   3.1.1 Synthesis of Base Polymers ..........................................................................................27  
   3.1.2 Incorporation of CGRGDS Peptides by Chain Transfer .............................................27  
   3.1.3 Incorporation of Peptides by N-Hydroxysuccinimide (NHS) Chemistry .....................28  
3.2 Polymer Characterization ........................................................................................................30  
   3.2.1 Composition Analysis ....................................................................................................30  
   3.2.2 Molecular Weight Analysis .........................................................................................30  
   3.2.3 Water Absorption .........................................................................................................30  
   3.2.4 Static Contact Angle .....................................................................................................31  
   3.2.5 Peptide Synthesis and Amino Acid Analysis .................................................................31  
   3.2.6 Fibrinogen and Albumin Adsorption ..........................................................................32  
   3.2.7 Preparation of Film Surfaces for Cellular Adhesion and Growth Studies ....................33  
   3.2.8 Preparation of Fibrous Surfaces for HUVEC Adhesion and Growth Studies .............33  
3.3 Cell Culture and Biological Assays ..........................................................................................34  
   3.3.1 HUVEC and EPC Culture ............................................................................................34  
   3.3.2 Adhesion and Growth of HUVECs and Cord Blood ECFCs under Static Conditions .........................................................................................................................35  
   3.3.3 Morphology of HUVECs under Static Conditions .........................................................36  
   3.3.4 Flow Cytometry ............................................................................................................37  
   3.3.5 Cell Viability and Metabolism ......................................................................................37  
3.4 Cell Adhesion Protocol under Flow Conditions .....................................................................38  
   3.4.1 Radial Flow Chamber ..................................................................................................38  
   3.4.2 Cell Staining Procedure for Adhesion on Fibrous Scaffold .........................................40  
3.5 Statistical Analysis ....................................................................................................................41
4 THE ADHESION OF HUVECS AND CORD BLOOD ECFCs ON RGD LINKED PEGYLATED POLYMERS UNDER STATIC CONDITIONS......................................................42

4.1 Introduction .......................................................................................................................42
4.2 Experimental Methods ......................................................................................................44
4.3 Polymer Characterization ..................................................................................................45
  4.3.1 Polymer Composition and Molecular Weight ........................................................45
  4.3.2 Interactions of PEGylated Terpolymer with Water.................................................48
4.4 Biological Interactions ......................................................................................................49
  4.4.1 Protein Adsorption ..................................................................................................49
  4.4.2 Assessment of cell adhesion ...................................................................................50
  4.4.3 Polymer Processing ................................................................................................54
4.5 Summary ...........................................................................................................................58

5 THE ADHESION OF HUVECS, CORD BLOOD ECFCs AND HBOECS ON POLYMERS CONTAINING CELL SPECIFIC BINDING PEPTIDE ................................................................59

5.1 Introduction .......................................................................................................................59
5.2 Experimental Methods ......................................................................................................62
5.3 Polymer Synthesis and Characterization...........................................................................63
5.4 Biological Assays ..............................................................................................................65
  5.4.1 Endothelial Outgrowth Cells from Adult Peripheral Blood ................................65
  5.4.2 Metabolic Activity ..................................................................................................66
  5.4.3 HBOEC and HUVEC Adhesion to Polymer Films .............................................67
  5.4.4 Cell Morphology ....................................................................................................69
5.5 Summary ...........................................................................................................................71

6 EFFECT OF PEPTIDE DENSITY AND PEG CONTENT ON CELL ADHESION AND PROLIFERATION........................................................................................................73

6.1 Introduction .......................................................................................................................73
6.2 Experimental Methods ......................................................................................................75
6.3 Polymer Characterization ..................................................................................................76
6.4 The Adhesion of Endothelial Cells under the Static Conditions ....................................78
  6.4.1 The adhesion of Cord Blood ECFC on Polymer Surfaces ....................................78
  6.4.2 Cord Blood ECFC Morphology ..........................................................................81
  6.4.3 The Adhesion of HUVEC on Polymer Surfaces ................................................83
6.6 The Adhesion of Cord Blood ECFCs under Flow .............................................................84
6.6 Summary ...........................................................................................................................86

7 THE ADHESION OF HUVEC, CORD BLOOD ECFC AND HBOEC ON RGD LINKED POLYMERS UNDER FLOW CONDITIONS................................................................................87

7.1 Introduction .......................................................................................................................87
7.2 Experimental Methods ......................................................................................................89
7.3 Polymer Characterization ..................................................................................................90
7.4 Biological Interactions ......................................................................................................91
  7.4.1 Adhesion Pattern and the Effect of Lifting Conditions .........................................91
  7.4.2 Dynamic Adhesion of HUVECs and Cord Blood ECFCs to Unfunctionalized Polymer Films..........................................................93
  7.4.3 Dynamic Adhesions of HUVECs and ECFCs to Functionalized Polymer Films...94
LIST OF TABLES

Table 2.1 Examples of Implant Tissue Interactions ................................................................. 12

Table 2.2 Peptides Identified from Phage Display Technology ............................................. 21

Table 2.3 Non-Fouling Surface Compositions ....................................................................... 22

Table 2.4 Some Common Polymers and Their Applications ............................................... 25

Table 4.1 Composition and Molecular Weight Averages of Polymers Studied .................... 47

Table 4.2 Interactions of PEGylated Terpolymer with Water ............................................... 48

Table 5.1 Composition and Molecular Weight Averages of Polymers studied .................... 63

Table 5.2 Interactions of PEGylated Terpolymer with Water ............................................... 64

Table 6.1 Composition and Molecular Weight Averages of Polymers studied .................... 77

Table 6.2 Interactions of PEGylated Terpolymer with Water ............................................... 78
LIST OF FIGURES

Figure 2.2 The Structure of Human Blood Vessel ................................................................. 16

Figure 2.3 Morphology of Endothelial Progenitor Cells ...................................................... 18

Figure 2.4 Structure of Cell Membrane ................................................................................. 20

Figure 2.5 Water Releasing and The Compression of The PEG Chain Due to Protein Deposition 24

Figure 3.1 Polymer Structure Using Chain Transfer ............................................................ 28

Figure 3.2 Polymerization Process Using NHS Chemistry ................................................... 29

Figure 3.3 Schematic Representation of Electrospinning Process ........................................ 34

Figure 3.4 Structure of the Radial Flow Chamber ............................................................... 40

Figure 4.1 $^1$H NMR Spectra of PEG Monomer, H20 and H20P15 ....................................... 46

Figure 4.2 Fibrinogen and Albumin Adsorption on Different Polymers ............................... 50

Figure 4.3 HUVEC Adhesion on Different Polymers at 2 hours. ........................................ 51

Figure 4.4 HUVEC and ECFC Adhesion on Different Polymers at 2 hours and 8 Days with medium containing 10% serum ........................................................................... 53

Figure 4.5 Fluorescence Images of HUVECs Adherent to Polymers After 2 Hours of Culture: .... 55

Figure 4.6 Scanning Electron Micrographs of Electrospun Polymers at $\times$300 Magnification: ...... 56

Figure 4.7 HUVEC Adhesion to Electrospun Polymer Scaffolds after 2 Hours of Incubation .... 57

Figure 5.1 Images of 4th Passage Adult HBOECs ................................................................. 66

Figure 5.2 Metabolic Activities of Adult HBOEC ................................................................. 67

Figure 5.3 the General Adhesion Motif RGD Increased Adhesion in All Cell Lines ............... 68
Figure 5.4 Confocal Micrographs (20X) of Adult HBOEC .............................................................70
Figure 5.5 Confocal Micrographs (20X) of Adult HUVEC .............................................................71
Figure 6.1 ECFC Adhesion on Polymers with High RGD Content ..................................................79
Figure 6.2 ECFC Adhesion on Polymers with Low RGD Content ....................................................80
Figure 6.4 HUVEC Adhesion on Polymers with High RGD Content .............................................83
Figure 6.5 HUVEC Adhesion on Polymers with Low RGD Content .............................................84
Figure 6.6 Dynamic Adhesion of Cord Blood ECFCs to Polymers and TCPS ....................................85
Figure 7.1 Real Time Shear Dependent Cord Blood ECFCs Adhesion to TCPS ............................92
Figure 7.2 Cord Blood ECFC Attachment on TCPS with Different Lifting Conditions after 10 Minutes ............................................................................................................................... .................................................................93
Figure 7.3 Dynamic Adhesions of HUVECs and Cord Blood ECFCs to TCPS and H2O after 10 Minutes .............................................................................................................................94
Figure 7.4 HUVEC Adhesion on Functionalized Polymer Surfaces after 10 Minutes ..................95
Figure 7.5 Dynamic Adhesions of HUVECs, Cord Blood ECFCs and HBOECs to Functionalized Polymer Films after 10 Minutes .........................................................................................96
Figure 7.6 Scanning Electron Micrographs of Polymer Fibers and Fluorescence Microscope Images of Cells Adherent on Fibers ............................................................................................97
Figure 7.7 Dynamic Adhesions of HUVECs, Cord Blood ECFCs and HBOECs to Functionalized Polymer Fibers after 10 Minutes at 5 s⁻¹ .......................................................................................98
CHAPTER 1

INTRODUCTION

1.1 Motivation and Objectives

Biomaterials are defined as synthetic materials or materials of natural origins which are placed into tissues or the biological environment with the aim to restore the function of a damaged organ without adversely affecting the live host [1]. Natural living tissues generally are capable for self-repair but due to the limited supply and immune rejection, artificial implants sometimes are the best option to meet the need of a human spare part [2, 3]. In recent years, the use of synthetic and natural materials for medical devices has advanced rapidly and biomaterials science has evolved into a multidisciplinary field that requires knowledge of polymer science, biochemistry, microbiology, immunology, mechanical engineering and physics, etc [4]. All of these disciplines are essential for the designing of the specific physical and chemical parameters required of biomaterials. Nowadays, more than 40 different materials are currently used to replace or repair a diseased or missing tissue, such as bone cements, breast implants, blood vessel prostheses, artificial hips and contact lenses etc. Due to the success of biomaterials and devices made from them, millions of patients have enjoyed pain relief and an improved quality of life [2]. However, most materials and implants currently available are limited in function and durability because they are not truly biocompatible.
In 1902, the initial publication of Alexis Carrel’s work opened an important sub-field of biomaterials, artificial blood vessels or vascular grafts. It was not until 1952 that Voorhees first used a synthetic graft to bridge arterial defects in dogs [5]. A few years later, Dacron arterial prostheses became commercially available. Like all other materials, problems exist with these cardiovascular biomaterial grafts. Biomaterial-induced thrombosis is one of the most difficult challenges. When exposed to blood contacting implants, serum proteins spontaneously adsorb to the surface, forming a protein layer. Platelets will spread on this protein layer and release their granular content resulting in further platelet aggregation. Thrombin is released and causes the stabilization of the platelet thrombus through the polymerization of adsorbed fibrinogen. In the case of injury, this process prevents the loss of blood and heals the wall of the vessel. In vascular grafts especially small diameter ones, this phenomenon causes the formation of thrombus which can result in failure of the graft [6, 7].

In the very early process of implantation, blood and material interactions begin with protein adsorption to the biomaterial surface. The success of a biomaterial may be determined by the ability of its surface to promote or inhibit certain protein or cellular responses. Many attempts have been made to improve blood interactions through surface modifications. Biotolerant or bioactive modifications are the two major strategies applied today [8]. Examples of biotolerant approaches are hydrogel coatings and poly (ethylene glycol) (PEG) immobilization. Examples of bioactive surface modifications are heparinization and the incorporation of peptides which
promote desired biological interactions [9, 10, 11]. In fact, the endothelium of native vasculature is the only existing truly blood compatible surface. The luminal surface of the human blood vessel is covered with a monolayer of endothelial cells. By seeding these cells on a surface, thrombogenic protein deposition and blood clotting should be prevented [12]. However, there are numerous problems with this approach. Seeded cells are not strongly attached and the endothelium often undergoes damage during implantation. Furthermore, those methods require isolation of the host’s endothelial cells and the pre-culturing of such cells on the material surface before implantation which are troublesome and time-consuming. Other problems include cell rejection of non-autologous cells used and the formation of neointimal hyperplasia [13, 14, 15].

The primary objective of this work was to design a biomaterial to capture circulating endothelial cells and that has the potential to generate a confluent functioning endothelium *in vivo* by the incorporation of PEG and novel peptides. Several novel peptides identified using a phage display technique were used to increase the binding affinity between the surface and endothelial cells. Polyethylene glycol was employed to limit protein adsorption and non-specific cellular interactions. PEG also worked to protect the immobilized peptides from the influence of the adsorbed serum proteins. Evaluating cell adhesion with functionalized polymer coatings under flow conditions provided valuable information preliminary to future in vivo implantation studies. Such knowledge can lead to strategies for designing materials and implant surfaces that can self endothelialized by harvesting mature endothelial cells or endothelial progenitor cells from the blood circulation and thus the
creation of an ideal biocompatible cardiovascular material.

Chapter 2 provides an overview of the most common problems associated with biomaterials and human blood vessel replacement. The mechanism of thrombus formation due to blood or serum exposure to foreign surfaces is discussed. A concise introduction to surface modification, including PEG and peptide incorporation, is also presented. The endothelium of native vasculature appears the only truly blood compatible surface. Both mature endothelial cells (HUVEC) and endothelial progenitor cells (EPC) were used in this research to endothelialize the surface of biomaterials. Details on the nature of HUVECs and EPCs are described as well as the phage display technology for the discovery of the novel peptides which specifically bind to EPCs. Finally, background on the methacrylate polymer system used is also mentioned in this Chapter.

Chapter 3 describes the materials and methods used in this work. Polymer synthesis and two different ways of incorporating peptides are presented and compared. Several polymer characterization techniques which were deployed in this research, such as NMR, GPC, contact angle, water adsorption and amino acid analysis, are described. Polymer processing methods which produced films and fibrous scaffold are also described. In order to characterize the cellular response under flow conditions, an automated video microscopy system combined with a radial flow chamber was used and detailed. Various biological assays including short and long term cell culture, a DNA quantification assay, a protein adsorption assay and cell staining are described.

In Chapter 4, HUVECs and Endothelial Colony Forming Cells (ECFCs)
adhesion onto a variety of surfaces in the presence and absence of serum proteins under static conditions are described. Polymer samples were synthesized through free radical polymerization and were cast into film samples or electrospun into a porous mat. Both chain transfer and succinimide chemistry were used to introduce peptide into the polymer. The advantage of succinimide chemistry was clear in terms of ease of reaction, maintenance of molecular weight and efficiency of peptide incorporation. To reduce the effect of serum protein adsorption, peptides were immobilized at the end of PEG side chains. Arginine-glycine-aspartate (RGD) was used as a cell adhesive positive control and arginine-glycine-glutamate (RGE) was introduced as a negative control. Polymers were characterized to confirm their composition. Cord blood ECFCs bought from Lonza and mature endothelial cells (HUVECs) purchased from ATCC (American Type Culture Collection) were the two cell types studied in this chapter. Both cell lines were cultured on films of peptide linked surfaces for varying time periods. Cell adhesion to electrospun fibrous structures containing peptides was also studied. The availability of peptides and the effect of serum proteins on cell adhesion were evaluated on random fiber meshes.

Chapter 5 deals with the adhesion of HUVECs, cord blood ECFCs and peripheral blood ECFCs which are also known as Human Blood Outgrowth Endothelial Cells (HBOECs), on surfaces incorporated with phage display identified peptides. GHM and TPS (Table 2.2) were two novel peptides which show specific binding affinity to EPCs. These peptides were incorporated into polymer films at the end of PEG spacer arms. Peripheral ECFCs were harvested from human blood and the
percentage of selected surface markers were characterized using flow cytometry. Several peptides including those novel specific peptides were tested with three cell sources (HUVECs, cord blood ECFCs and HBOECs). Cell morphology on surfaces was studied using confocal microscopy. Cell metabolic and viability assays were also conducted to further investigate the cellular responses to the polymer surfaces.

Chapter 6 describes efforts towards the optimization of the effect of surface properties on cell responses. PEG content and peptide density play an important role with respect to the initial adhesion and proliferation of cells. The effects of PEG and adhesive peptides work against with each other. PEG prevents adhesive protein deposition and thus attenuates cellular attachment while cell binding peptides promote cell adhesion. Polymers with a series of peptide densities and PEG content were synthesized. Long term and short term cell culture were conducted.

Chapter 7 describes how the presence of cell binding peptides affects cell adhesion under flow conditions. Polymer films were coated on a glass substrate by spin coating while fiber matrices were generated by electro-spinning. Video microscopy combined with a radial flow chamber allowed study of the effects of shear stress on cell adhesion. A computer program was written to control the video microscope stage automatically. In order to observe cells on the fiber mesh under flow, cells were tagged with fluorescent molecules. Cell attachment data were collected on several peptide linked surfaces and the adhesion characteristics of HUVECs and cord blood ECFCs were compared.

Chapter 8 summarizes the major accomplishments of this work and offers
recommendations for future research. Mathacrylates linked with peptides are excellent polymer substrates for the design of biocompatible materials. The interactions occurring between endothelial cells, polyethylene glycol and phage display peptides were studied in detail. Other functional monomers and cell binding peptides can also be incorporated to achieve desired functionalities. It is hoped 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 field who focus on the development of a new generation of biocompatible surfaces.
CHAPTER 2

LITERATURE REVIEW

2.1 Biomaterial

2.1.1 Introduction of Biomaterial

Biomaterials are synthetic or natural materials that are used in biomedical devices or biological systems to restore a body’s natural function. By the early twentieth century, advances in the science and technology of polymers and ceramics promoted widespread consideration of their use as implantable materials. Improved implants with better resistance to the corrosive implant environment were developed due to the advances in materials science and the demands of two world wars [1]. After a steady growth of over half a century, biomaterials have developed into a multidisciplinary field that requires knowledge of polymer science, biochemistry, microbiology, immunology, mechanical engineering and medicine, etc. [2].

Many different synthetic and modified natural materials are used in biomaterials, such as ceramics, metals, polymers, glasses, carbons and composites. The resulting materials are in the form of molded parts, coatings, fibers, foams and films. Biomaterials are primarily used for medical applications including implants, devices and prosthesis. In order to maintain special properties and biological functions, a single device generally uses many different materials. A heart valve may be fabricated from polymers and metals. A bone replacement might be produced from metals and
ceramics. Beside their wide use as implants, biomaterials are also used to grow cells in culture, to assay for blood proteins in the laboratory and in diagnostic gene arrays, etc. [1-4].

![Figure 2.1 Numbers of Medical Devices/yr. Worldwide (2004) [2]](image_url)

The number of biomaterial based implants used each year in humans has increased enormously since the 1960s due to better understanding of biomaterials interactions with tissue. The population suffering from age related disabilities is also increasing. Treating such diseases as osteoporosis will drive the demand for improved biomaterial products. The improvement in surgical skills and technological advances in prosthetics have increased the confidence of patients along with the demand for surgical appliances that use biomaterials. Nowadays, implants are used to relieve pain
and increase function for many millions of patients. Individuals are able to enjoy a prolonged quality of life thanks to the success of biomedical materials. Figure 2.1 estimates the numbers of medical devices per year produced worldwide [3,5,6].

Although great successes have been achieved with biomaterials, artificial materials are limited in durability and functionality, because they are not live and cannot replenish themselves. In order to increase their performance, it is essential to have the mechanical and chemical properties of biomaterials compatible with the host tissue. A biomaterial should also not be toxic to the surrounding tissue unless it has been designed for such a requirement. For example, some drugs may be released from biomaterial implants with the aim to destroy cancer cells. With international standards and regulations, most biomaterials perform satisfactorily today. Still, humans differ in life patterns, body chemistries and may react to implants differently. Additionally, infection remains a serious problem for implant recipients. Finally manufactured devices have a life span and endurance limits as well. These many factors encourage further research and development to ever improve biomaterials and implants made from them [2,3].

2.1.2 Implant-Tissue Interactions

It is inevitable that host tissue receives damage from either the implantation process or the incompatibility of the implant. Inflammation, a nonspecific response to tissue damage, will occur in physiological systems. The presence of incompatible material may cause persistent tissue changes due to mechanical or chemical
interactions. The consequences include trauma, local cell death and foreign body responses. It is highly desirable to understand these tissue responses due to the intrusion of foreign materials, so that they may be controlled and prevented for improved function in vivo [3,7].

Wound healing and inflammation occur during implantation and are controlled by effects in the connective tissue. Damaged tissue and bacteria release chemicals, which cause the blood vessels to dilate and increase the permeability of their endothelial cell linings. This produces the characteristic signs of inflammation, seen as reddening, warmth and swelling. The first stage of healing process after implantation involves the leakage of water and proteins to the surrounding tissue bed. The next stage is about the fibroblast proliferation and the final stage is the remodeling of the tissue inside or around implants depending on the types of materials [7].

The biocompatibility of materials has increasingly drawn the attention of engineers dealing with biological problems. Besides traditional design aspects such as selecting a biomaterial’s physical properties, researchers have to consider a material-biological interaction. The tissue response allows materials to be classified into four main categories (Table 2.1). Living systems exhibit change in the presence of implants and a material may or may not be biocompatible in different patients or applications. With an understanding of the tissue response, the biomaterials scientist may be able to select a material that will fit optimally in certain situation [3,7,8,9].
<table>
<thead>
<tr>
<th>Types of Materials</th>
<th>Response of Surrounding Tissues to Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic</td>
<td>Cells and surrounding tissue are killed which is unacceptable in any implant applications unless such effect is intentional or can be controlled.</td>
</tr>
<tr>
<td>Inert</td>
<td>A fibrous capsule which is non-adherent will occur. The thinner the capsule, the more successful the material.</td>
</tr>
<tr>
<td>Bioactive</td>
<td>A capsule which is adherent will occur. There is minimal encapsulation.</td>
</tr>
<tr>
<td>Biodegradable</td>
<td>The material will be replaced by host tissue gradually.</td>
</tr>
</tbody>
</table>

Table 2.1 Examples of Implant Tissue Interactions [3]

2.1.3 Vascular Grafts

The work presented in this dissertation focuses on an important subclass of biomaterials, which is designed to contact blood. The death rate from cardiovascular diseases is rising rapidly in developing countries because of increasing life spans. As individual ages, the deposition of fat and tissue debris in the wall of blood vessels may block the blood circulation and cause serious disease. The increase in cardiovascular disease has accelerated the development of materials to be used in contact with blood. Typical examples for such applications are blood vessel replacement and artificial heart valves [10, 11]. Specifically, the novel biomaterial developed in this research is intended for use in small diameter vascular graft
applications.

Both biological or synthetic blood vessel replacements exist today and each of them have advantages and disadvantages. Biological grafts can be classified as either autograft or allograft. An autograft is using a patient’s tissue taken from another site. The most commonly used such graft in peripheral vascular surgery is the saphenous vein. Another example of an autograft is used in the coronary artery bypass surgery and involves using a mammary artery from the patient’s chest. Even though autografts are often preferred as there is no concern of foreign body rejection, their limited availability has become a large barrier for wide usage. An allograft is using tissue from another person or animals. Externally supported umbilical vein is rarely used due to the long term need of immunosuppressant drugs and potential disease contamination. Most allografts are decellularized to prevent the immune responses [3, 12].

Voorhees started to develop synthetic vascular grafts in the 1940s and the first one used in vivo was made in 1952 from Vinyon "N". It was implanted in a dog’s aorta and coated with a smooth antifouling surface to reduce the formation of thrombus [13]. Later efforts to modify human and other mammalian vascular replacements include decellularization, freeze drying, etc. However due to the mechanical mismatch between the graft and blood vessel and the generation of aneurysm, all of them were unsuccessful. A desirable vascular graft should first of all resist non specific protein and cellular deposition or be relatively bio inert. It is also valuable to fabricate materials used for vascular applications into scaffolds with adequate pore
distributions which will permit fibroblast reorganization and improve the encapsulation of the surrounding tissue. Great success was achieved with large diameter vascular grafts (>6mm) where high flow rates and low resistance apply. Occlusion of the vascular replacement much earlier occurs when the diameter is small (<6mm). To prevent the need for multiple surgeries and potential death, improvement in current vascular graft materials and constructs are required [14].

2.1.4 Problems with Current Vascular Graft

Today, synthetic grafts are most commonly made from Dacron or polytetrafluoroethylene (PTFE). Vascular grafts with a diameter larger than 6mm are clinically very successful. In large diameter grafts the thrombus is stabilized by fibrin and the cross sectional area of the graft will not be completely occluded. Approximately 90% of large diameter grafts can survive for 5 - 10 years or longer. The primary area of failure in prosthetic grafting with Dacron and PTFE is that the patency of grafts with a diameter less than 6 mm remains poor. About 50% of small diameter vascular grafts lose functionality after 5 years of implantation. Primary factors that affect these prosthetic grafts include inadvertent activation of the body’s coagulation response as well as the formation of neointimal hyperplasia (thickening of a blood vessel as part of the tissue remodeling response). Thus it would be highly desirable to develop a scaffold which enables blood contact without significant thrombosis [15].

When blood contacts artificial material, protein and platelets deposition will
occur. Thrombin is released and causes the stabilization of the mass through the polymerization of adsorbed fibrinogen. In the case of injury, this phenomenon prevents the loss of blood and begins the hearing of the wall of the vessel. In small diameter vascular grafts, this phenomenon causes the formation of thrombus which typically results in failure of the graft. The goal of this thesis was to develop a material system that would prevent the formation of thrombosis in a small diameter artificial blood vessel for long term implantation [2, 16-19].

The luminal surface of human blood vessel is covered with a monolayer of endothelial cells (ECs). Figure 2.2 shows the structure of human blood vessel. It has been shown that these cells resist blood clotting and prevent thrombogenic protein deposition.

The seeding of prosthetic grafts with endothelial cells and subsequent proliferation of these cells into a confluent monolayer along the graft has been suggested as an attractive solution to graft thrombogenicity. However in humans all synthetic grafts and modified grafts of biologic origin fail to develop an endothelial lining. The problem is that the endothelium is delicate and often undergoes damage during implantation. The seeded cells are weakly adherent to the surface and the formation of anastomotic hyperplasia, mostly consisting of smooth muscle cells, reduces flow gradually. This leads to retrograde thrombosis and failure of the graft. In order to improve the function of small diameter vascular replacements, scientists are seeking other ways. In some animal models, endothelial cells in the blood can adhere or migrate to endothelialize a vascular graft by themselves [27]. Unfortunately this
does not occur in humans. A possible alternative is to employ other circulating endothelial cell resources as a new strategy to endothelialize a luminal surface in humans in vivo [14].

2.2 New Strategy to Tailor the Interface

2.2.1 Endothelial Progenitor Cells

Recently a particular group of cells has been discovered with great clinical potential, which are known as Endothelial Progenitor Cells (EPCs) (Figure 2.3). These are bone-marrow derived cells that have the capacity to differentiate into endothelial cells. The first evidence indicating the presence of EPCs in the adult circulation emerged when a small subset of mononuclear blood cells was shown to acquire an endothelial cell-like phenotype in-vitro [21]. Further studies showed these cells possessed both expression of CD34 and vascular endothelium growth factor receptor-2 (VEGFR-2) [22]. EPCs have been successfully applied to the field of tissue engineering as a means of improving biocompatibility of vascular grafts. In a canine
model, artificial grafts, first seeded with autologous CD34 cells from bone marrow and then implanted into the aorta were found to have increased surface endothelialization and vascularization compared to controls [23].

There are two distinct populations of EPCs that have been discovered: early outgrowth colony-forming cells and late outgrowth colony forming cells (ECFCs) [9]. Late outgrowth ECFCs are more endothelial cell like and they can be harvested from both peripheral and cord blood. Peripheral blood ECFCs are also called human blood outgrowth endothelial cells (HBOECs). Cai et al. [24] found that colony-forming progenitor cells obtained from cord blood (cord blood ECFCs) emerge at later time points in culture and have undergone endothelial differentiation, but appear to still possess functional properties different from mature endothelial cells. Peripheral blood ECFCs/HBOECs can be grown reproducibly as monolayers from human peripheral blood buffy coat mononuclear cells. These cells have typical endothelial cobblestone morphology and express multiple endothelial cell markers including CD34, von Willebrand factor (vWF), and CD31, etc. Lin et al. [25] have reported that these cells can be expanded 5000 times from 65 days after the start of culture.

Yoon et al. [26] have compared the two types of cells that can be cultured from human peripheral blood, early endothelial progenitor cells (EPCs) and outgrowth endothelial cells (HBOECs). Both CD14(+) and CD14(-) cells derived from monocytes are confirmed as the surface markers for early endothelial cells. It is reported that outgrowth endothelial cells (HBOECs) are cultured almost exclusively from CD14(-) cells, not CD14(+) cells. HBOECs are distinct from mature
endothelial cells like HUVEC. Yoon found early EPCs and a portion of CD14(-) cells, which might be HBOECs, proliferate rapidly and are distinct from mature endothelial cells in terms of kinase insert domain receptor (KDR) expression level.

Both HBOECs and cord blood ECFCs possess higher proliferation potential and produce fewer growth factors than early outgrowth cells and thus are less likely to promote neointimal hyperplasia [23]. These findings suggest the use of ECFCs as a suitable source of cells for constructing functional tissue engineered blood vessel replacements. However, those advantages are offset by the current complex and time consuming isolation procedures required to obtain ECFCs especially from peripheral blood. An alternative strategy is to develop materials that self endothelialize by harvesting ECFCs from the circulation. We propose to covalently incorporate peptides identified through phage display technology, which can specifically bind to EPCs, into a methacrylate polymer matrix. The goal is to enhance endothelial cell coverage of the biomaterial.
2.2.2 Peptides

Cell binding occurs between cell membrane proteins and the external environment. To facilitate endothelial cell attachment researchers have exploited the molecular biology of the cell membrane. Biological membranes can be considered as a two-dimensional liquid. All lipid and protein molecules diffuse fairly on the membrane surface [28]. Indeed the membrane surface consists not only of the lipid bilayer but it also contains a large quantity of proteins, which provide communication and adhesion channels between cells and the environment (Figure 2.4). These protein molecules can be found on the inside and outside of the cell membrane. Some of them are so large to span the entire membrane. These proteins are essential for cell adhesion, endocytosis, exocytosis and communication. There are various types of proteins including integrins, desmosomes, cadherins, caveolaes, and different structures involved in cell function [29, 30]. The integrin superfamily of receptors is responsible for most cell-environment attachments. Integrins contain two distinct chains, called α (alpha) and β (beta) subunits. As receptors for ECM proteins, integrins provide anchorage and convey signals that regulate cell growth, differentiation and migration. Although the molecular mass of the integrin subunits can vary from 90 kDa to 160 kDa, many share certain amino acid sequences in common which act as binding sites or receptors for integrin mediated cell attachment [31]. Among the adhesive protein ligands, the active peptide sequence arginine-glycine-aspartate (RGD) which is found in fibrinogen and fibronectin has been most studied [32, 33].
Advances in cell biology and the identification of binding peptide sequences, such as the RGD tripeptide, have stimulated the development of bioinspired cell adhesive materials. In order to produce biofunctional surfaces, several strategies may be applied, such as the incorporation of short adhesive peptides onto appropriate synthetic materials and the immobilization of non-fouling and non-adhesive molecules to reduce background effects [34]. Several groups have demonstrated that incorporation of RGD peptides into synthetic polymers and silicon based substrates promotes cell adhesion and migration which is mediated by cell integrins [35, 36]. Although this biomimetic approach remains one of the most promising strategies, its practical utility may be limited by the need to design materials that are optimized to promote cell adhesion of a specific cell type. To address this need we propose to use peptide ligands that have been selected by phage display screening and have been shown to bind specifically and with high affinity to blood outgrowth endothelial progenitor cells.

The use of peptide ligands for specific cell types promises to be a valuable tool both in research and clinical applications [37]. The invention of phage display
technology has proven to be an extraordinary way for discovering high affinity new peptide ligands. The technique of phage display was first reported in 1985 by Dr. George Smith [38]. 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 *E. coli*. The DNA encoding the protein or peptide of interest is ligated into the gene to achieve display. The inserted DNA sequence can be randomized to create a library of phages. By incubating the library with a target of interest, washing out weak or non-binders, and repeating the process allows for the enrichment of tight binders. This is a processes referred to as bio-panning [39].

Veleva and Cooper performed non-biased selection using an outgrowth of endothelial progenitor cells (HBOECs), which is a type of EPCs, as an affinity matrix [19]. To avoid non-specific binding, they applied a negative-positive selection approach by pre-incubating the library with mature endothelial cells (HUVEC). Several 12-mer peptide ligands that bind to HBOECs were isolated (Table 2.2). Among them, the peptide with the highest binding affinity was TPSLEQRTVYAK (abbreviated as TPS) [19]. After incorporation to a biomaterial, the TPS peptide ligands should assist the binding of EPC-derived endothelial cells from the blood stream.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHMDMSPHAVID-GGGSC</td>
<td>GHM</td>
</tr>
<tr>
<td>SYQTLKQHLPYG-GGGSC</td>
<td>SYQ</td>
</tr>
<tr>
<td>TPSLEQRTVYAK-GGGSC</td>
<td>TPS</td>
</tr>
</tbody>
</table>

*Table 2.2 Peptides Identified from Phage Display Technology*
2.2.3 Poly Ethylene Glycol

After adsorbing to the surface of materials, proteins will undermine the effectiveness of specific peptide ligands, since there are so many binding domains on the serum proteins. In this thesis anti-fouling molecules were introduced to limit those non-specific interactions [41]. It is generally acknowledged that hydrophobic surfaces tend to adsorb proteins due to the entropic gain and enthalpy loss. It is also known that cationic proteins will bind to anionic surfaces and vice versa due to cation-anion attractive interactions [42]. Table 2.3 lists several non-fouling surface compositions. The most common approaches to creating non-fouling surfaces are to make them hydrophilic and electrically neutral [6].

<table>
<thead>
<tr>
<th>(A) Synthetic hydrophilic surfaces</th>
<th>PEG polymer and surfactants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutral polymers</td>
</tr>
<tr>
<td></td>
<td>Phosphoryl choline polymers</td>
</tr>
<tr>
<td></td>
<td>Gas discharge-deposited coatings</td>
</tr>
<tr>
<td></td>
<td>Zwitterionic polymers</td>
</tr>
<tr>
<td>(B) Natural hydrophilic surfaces</td>
<td>Passivating proteins</td>
</tr>
<tr>
<td></td>
<td>Polysaccharides</td>
</tr>
<tr>
<td></td>
<td>Liposaccharids</td>
</tr>
<tr>
<td></td>
<td>Phospholipid bilayers</td>
</tr>
<tr>
<td></td>
<td>Glycoproteins</td>
</tr>
</tbody>
</table>

Table.2.3 Non-Fouling Surface Compositions

Poly (ethylene glycol) (PEG) is the one of the most commonly used non-fouling material. The idea of PEGylated materials was proposed by Frank Davis in the 1960s.
Researchers began to use PEG grafted surfaces for the prevention of protein deposition from the 1980s [43]. For years, PEG's nonfouling surface properties were mostly credited to its steric repulsion effect. In aqueous solution, water-soluble proteins tend to have their hydrophobic residues buried in the middle of the protein, whereas hydrophilic side chains are exposed to the water molecules [44]. As shown in Figure 5, when a hydrated protein molecule is adsorbed to the surface, water molecules associated with the PEG surface are released into the bulk solution. This will lead to an increase in enthalpy (+ΔH_{waterloss}) due to protein and surface dehydration, which is enthalpically unfavorable [45]. The dehydration process is also accompanied with a system entropy gain due to the destructuring of the ordering of the water molecules around the chain, (+ΔS_{waterloss}) which makes the protein deposition on PEG materials entropically favorable. This entropy loss is offset by the compression of the PEG molecules. From Figure 2.5, not only the water is released, but also the coil of PEG chains is compressed during the protein adsorption process (-ΔS_{pegcompress}). In order to determine the system entropy, both water releasing and PEG compression effects have to be considered [46,47,48].

In most cases, the overall free energy change (ΔG_{system}) defined by equation 1 is positive and unfavorable [46]. Thus, the incorporation of PEG will generate a surface which reduces non specific protein deposition. There are other interaction forces between PEG molecules and proteins including Van der Waals steric repulsion, van der Waals attraction, and the hydrophobic interaction. In some special conditions, the situation may be reversed and the surface may favor protein adsorption.
\[ \Delta G_{\text{system}} = \Delta H_{\text{waterloss}} - T^* (\Delta S_{\text{waterloss}} - \Delta S_{\text{PEG compress}}) \]  

Another non-fouling strategy includes the incorporation of zwitterions such as phosphorylcholine and the immobilization of heparin, which is well known for its anti-thrombogenicity [49].

![Figure 2.5 Water Releasing and The Compression of The PEG Chain Due to Protein Deposition](image)

**2.3 Methacrylate Polymers**

Polymers are used widely for medical devices and implants. Early medical devices were based on high purity grades of commonly used industrial polymers. In recent years, new polymers have been specially synthesized for medical uses. Table 2.4 gives some common examples of synthetic polymer used in healthcare and their applications.

In this work, we chose to use acrylic copolymers comprised of various ratios of hexylmethacrylate (HMA), methylmethacrylate (MMA), and poly(ethylene glycol) methacrylate (PEGMA). Methacrylates, which resist biodegradation are useful for fabricating a small diameter artificial blood vessel. They have been used in the
biomedical area for many years and have been classified as bioinert [50,51]. Heath and Cooper [41] used hexylmethacrylate, methylmethacrylate, and methacrylic acid (20 mol%: 78 mol %: 2 mol %) to produce oriented and random fiber structures by electrospinning. They demonstrated that the electrospun methacrylates, especially with random fibers, significantly improved the cytocompatibility of the surfaces to endothelial cells. An advantage of methacrylate based materials is that their mechanical properties are easily modified by manipulating the glass transition temperature (Tg). After selecting appropriate monomers, one can tune the chemical nature of the backbone which impacts the Tg and thus the physical properties of the polymer. Another advantage of methacrylate polymer is that the resulting polymer is thermoplastic. Thermoplastic polymers can be molded to shape by the application of heat and pressure and can be reshaped once formed. Thermoplastic polymers can be further processed using electrospining into different 3D topographies which may impact cell adhesion and proliferation.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly (methylmethacrylate)</td>
<td>Contact lenses, bone cement</td>
</tr>
<tr>
<td>Polyethylene terephthalate</td>
<td>Artificial arteries</td>
</tr>
<tr>
<td>Polyurethanes</td>
<td>Catheters</td>
</tr>
<tr>
<td>Poly(propylene)</td>
<td>Sutures, heart valves</td>
</tr>
<tr>
<td>Poly(glycolide)</td>
<td>Biodegradable sutures</td>
</tr>
</tbody>
</table>

Table 2.4 Some Common Polymers and Their Applications [6]

In conclusion, this thesis describes an effort to design a methacrylate based
polymer to capture circulating ECFCs. The incorporation of PEG and high affinity ECFC/HBOEC binding peptides offers the potential to produce a methacrylate based material capable of growing a monolayer of endothelial cells in vivo.
CHAPTER 3
MATERIALS AND METHODS

3.1 Polymer Synthesis

3.1.1 Synthesis of Base Polymers

Base polymer was synthesized from 20 mol% hexyl methacrylate (HMA, Alfa Aesar, Ward Hill, MA) and 80 mol% methyl methacrylate (MMA, ACROS Organics, Pittsburgh, PA). PEGylated polymer was copolymerized from 20 mol % HMA, 65 mol% MMA and 15 mol% poly (ethylene glycol) methacrylate (PEGMA, Poly Sciences, Warrington, PA). The average molecular weight of PEGMA was 500. Copolymerization took place in N,N-dimethylformamide (DMF) with 2,2-azobisisobutyronitrile (AIBN, Sigma-Aldrich, Milwaukee, WI) as the free radical initiator. The reaction proceeded for 2 days as previously described [1]. At the end of the reaction, a 1:1 methanol: distilled water mixture precipitated the polymer. The precipitate was collected, dried and weighed.

3.1.2 Incorporation of CGRGDS Peptides by Chain Transfer

In a single step reaction, the peptide was polymerized with 20 mol% HMA, 65 mol% MMA and 15 mol% PEGMA. A oligo-peptide chain transfer agent was used, CGRGDS (Commonwealth Biotech Inc., Richmond, VA). Some of the polymer chain
ends were terminated by the peptide (Figure 3.1).

![Figure 3.1 Polymer Structure Using Chain Transfer](image)

### 3.1.3 Incorporation of Peptides by N-Hydroxysuccinimide (NHS) Chemistry

In a two step reaction, the PEG monomer is first combined with the peptide (Figure 3.2). The average molecular weight of acrylate-PEG-N-hydroxysuccinimide (JenKem Technology USA Inc., Allen, TX) was 2000. The reaction was conducted at a molar ratio of 1:1.2. To prevent the production of acryloyl-PEG-OH, the product of the competing hydrolysis reaction, anhydrous dimethylformamide (DMF) was used as the solvent. Unacrylated peptides and hydroxysuccinimide were separated from the desired product by dialysis (MWCO 2000, Thermo Fisher Scientific, Rockford, IL)
against de-ionized water for 24 h with periodic bath changes. The final acrylate-PEG-peptide monomer was lyophilized and stored at -20°C until use. In the second step, 20 mol% HMA, 65 mol% MMA, 14.8 mol% PEGMA and 0.2 mol% acrylate-PEG-peptide were polymerized with AIBN. The reaction proceeded for 2 days and the polymer was collected by precipitation in DI water. The chemical structure was confirmed by NMR and the peptide content in the conjugate by amino acid analysis.

Figure 3.2 Polymerization Process Using NHS Chemistry

In this dissertation, the polymer is referred by the mole percent of the monomer and the composition MMA is omitted in the terminology. H20 refers to a base material synthesized from 20/80 mol % HMA/MMA. H20P15 refers to a material copolymerized from 20/65/15 mol% HMA/MMA/PEGMA. H20P15RGD refers to the RGD containing H20P15 material with the peptide incorporation accomplished by chain transfer. H20P15NHSRGD refers to a polymer with the peptide incorporated by NHS chemistry, which was copolymerized from 20/65/14.8/0.2 mol%
HMA/MMA/PEGMA/ acrylate-PEG-RGD.

3.2 Polymer Characterization

3.2.1 Composition Analysis

The composition of the base polymer and the PEGylated polymer was determined through $^1$H Fourier transform nuclear magnetic resonance (NMR) spectroscopy. 400µl of 0.05g/ml polymer in deuterated chloroform (Sigma) was analyzed using a Bruker DPX 400 MHz NMR machine at 300K. The peptides and the NHS molecules comprise only 0.1 mol % of the reactant. The detection of those components is beyond the sensitivity of the technique.

3.2.2 Molecular Weight Analysis

Polymer molecular weight was analyzed through gel permeation chromatography (GPC). The preparation of the polymer sample and the procedure were reported by Heath and Cooper [3]. The NHS containing polymers were not soluble in THF. Thus, only the molecular weights of polymers without NHS are reported.

3.2.3 Water Absorption

The polymer was cut into samples weighing 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 Kim Wipe, and weighed after
30 min, 24 h and 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.

**3.2.4 Static Contact Angle**

The polymer was coated onto a glass disk of 2 mm diameter by dip coating and dried under vacuum overnight. Polymer films were pre-equilibrated with water for 3 hours. A 2 µL droplet of deionized water was pipetted onto the polymer 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 (Bethesda, MD).

**3.2.5 Peptide Synthesis and Amino Acid Analysis**

CGRGDS and CGRGES were purchased from Commonwealth Biotech, Inc, (Richmond, VA). 12-mer peptides were supplied by Commonwealth Biotech, Inc. or Dr. Pravin Kaumaya of the Ohio State University College of Medicine using standard solid phase peptide synthesizers. A triple glycine spacer was added to separate the potential cell binding ligand from the reactive cysteine terminus. The dodecamer peptides were previously isolated by their avid binding to “human blood outgrowth endothelial cells (HBOEC)” but not HUVEC [4, 5].

The amount of peptide incorporation was determined from amino acid analysis performed by Commonwealth Biotech, Inc. From our previous research [6], densities
of RGD above 2 µmol per gram polymer promoted robust HUVEC adhesion.

3.2.6 Fibrinogen and Albumin Adsorption

The culture surface of a 96-well plate was coated with the polymer of interest through the addition of 35 µL of a 5% polymer solution (g/ml) in 1:1 Acetone:DMF. The well plate was covered in aluminum foil and dried slowly overnight followed by drying in an oven and vacuum oven for 24 h each at 55–60ºC. The wells were equilibrated in PBS overnight at 37ºC. The PBS was aspirated, and 50 µL of a 50 µg/ml solution of fluorescently tagged fibrinogen and (Alexa Fluor 488 conjugated fibrinogen, Invitrogen, Carlsbad, CA) was added to each well. The protein solution was allowed to contact the surface for 40 min at 37ºC before the solution was aspirated, and the wells were washed 5 times with PBS to remove reversibly adsorbed fibrinogen. Fifty microliters 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. Experiments with fluorescently tagged albumin (Alexa Fluor 488 conjugated albumin, Invitrogen, Carlsbad, CA) were conducted similarly.
3.2.7 Preparation of Film Surfaces for Cellular Adhesion and Growth Studies

Polymer samples were dissolved in 1:1 Acetone:DMF at 5 wt%. Spin coating was performed in air by flooding the substrate surface with the solution (passed through a 0.2-µm filter) and spinning at 3,500 r.p.m. for 45 s. The films were then immediately dried in air for 5 min. Before contacting with cells, films were equilibrated with PBS for 1 hour.

3.2.8 Preparation of Fibrous Surfaces for HUVEC Adhesion and Growth Studies

Polymer samples were dissolved in 1:1 volume ratio Acetone:DMF mixture at 5 wt%. The polymer solution was fed by a syringe pump (Harvard Apparatus) into a steel capillary (I.D. = 0.047”) suspended vertically over the center of an aluminum collector plate (Figure 3.3). A combination of the three high-voltage generators (Gamma High Voltage Research) was employed with a high positive voltage (15 kV) to charge the steel capillary containing the polymer solution. A negative voltage (-10kV) was used to charge the aluminum collector plate. The polymer solution was fed at a rate of 3 ml/h by the syringe pump. The component spacing and applied voltage were optimized to provide controlled deposition of fibrous mats. The fibrous mats were allowed to dry overnight and then placed under vacuum for 48h at room temperature.
3.3 Cell Culture and Biological Assays

3.3.1 HUVEC and EPC Culture

HUVECs were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured according to standard procedures. Briefly, DMEM medium was supplemented with 0.1 mg/mL heparin (Sigma-Aldrich), 0.05 mg/mL endothelial cell growth supplement (Sigma-Aldrich), and 10 vol % fetal bovine serum. Cultures were incubated in a humidified environment at 37°C and 5% CO2. The culture medium was changed every 2 days, and cultures were passaged at 80% confluence to prevent contact inhibition. Passages 4 through 6 were used.

Cord blood Endothelial Colony Forming Cells (ECFCs) were purchased from Lonza (Switzerland). Cells were cultured according to standard procedure. EGM-2
medium was supplemented with EGM-2 SingleQuots (Lonza) and 10 vol % fetal bovine serum. The cells were seeded onto collagen coated T flasks. Cultures were incubated at 37 °C with 5% CO₂ and passaged at 80% confluence with 0.05% Trpsin/EDTA. Passages 4 through 6 were used.

Adult peripheral blood was donated at the Ohio State Medical Center with the approval of an appropriate institutional review board. Buffy coat mononuclear cells were obtained from 30-50 ml fresh adult peripheral blood by density gradient using Lymphocyte Separation Medium (Mediatech, Inc., Manassas, VA) according to the manufacturer's recommendations. The mononuclear fraction was resuspended in supplemented EGM-2 medium and seeded into collagen-coated well plates at a density of 1*10⁶ ml⁻¹. Nonadherent cells were removed after 48 hours and medium was replaced every two days thereafter until the appearance of colonies with cobblestone-shaped cells. Colonies were split at 80% confluency. HBOECs were used between the 3rd and 5th passages. For all cell adhesion and growth experiments, cells were lifted using 0.1mM EDTA w/o trypsin except where noted.

3.3.2 Adhesion and Growth of HUVECs and Cord Blood ECFCs under Static Conditions

The growth surface of a chamber slide was coated with 500µL of a 5% solution of the desired polymer in 1:1 v:v Acetone:DMF mixture, covered with aluminum foil, allowed to dry slowly for 24 h at ambient conditions to minimize bubble formation, and then dried in an oven at 55–60 °C for 24 h to remove residual solvent. Chamber
slides were sterilized through exposure to UV radiation for 2 h and equilibrated overnight in PBS before the introduction of cells. 1.5 ml of medium containing 75,000 cells was added to each well of the chamber slide (17,857 cells/cm²) and incubated for the desired length of time. Unattached cells were removed by three washes with phosphate buffered saline (PBS). The test surfaces were then covered with 1.5 ml of papain (Sigma-Aldrich) in an oven at 55–60°C for 24 h. Papain digested the cell membrane allowing the release of the cell's DNA. A total of 0.05 ml of cell lyses from each chamber was then transferred to three wells of a 96-well plate. A total of 0.05 ml of a 5 µg/ml solution of Pico Green® (Invitrogen) was added to each well and allowed to incubate for 5 min under ambient conditions. 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.

3.3.3 Morphology of HUVECs under Static Conditions

To image cell morphology on the scaffolds, samples were rinsed with PBS, fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with rhodamine phalloidin (Molecular Probes) for F-actin and DRAQ-5 (Biostatus Ltd., United Kingdom) for nuclei. Images were taken on a Zeiss 510 META laser scanning confocal microscope.
3.3.4 Flow Cytometry

Aliquots of 500,000 cells in 80 µl PBS containing 5% BSA were stained with 10 µl each of CD32 FcR blocker (StemCell Technologies, Vancouver), FITC CD34 (Miltenyi), PE CD133 (Miltenyi), and APC VEGFR-2 (Miltenyi). Isotype controls included APC IgG1, FITC IgG2a, and PE IgG (Miltenyi). Cells were incubated for 45 minutes at 4 °C then washed twice with 0.5 ml PBS (1% BSA). Flow cytometry (BD LSR II) was conducted on live cells shortly after staining.

3.3.5 Cell Viability and Metabolism

Enzymatic activity was assayed with Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) to assess viability. This colorimetric assay measures the cleavage of the metabolite testrasodium salt (WST-8) by mitochondrial dehydrogenase. The formation of formazan dye was measured by absorbance at 450 nm in a plate reader. Approximately 20,000 HBOECs and HUVECs were seeded into culture wells of a 24-well plate coated with the peptide-laden terpolymer and then incubated four hours at 37 °C. The supernatant was changed with fresh medium containing 10 vol% CCK-8 reagent. Cells were incubated a further four hours then dosed with a 10 vol% equivalent of of 1% sodium lauryl sulfate and stored at 4 °C until the absorbance was measured @ 450 nm. Supernatants from the 24-well plate were transferred to the wells of a transparent 96-well plate. Experiments were conducted in duplicate and measured three times. Wells containing only medium and CCK-8 reagent served as background.
3.4 Cell Adhesion Protocol under Flow Conditions

3.4.1 Radial Flow Chamber

Dickinson and Cooper described a radial flow cell to study the adhesion of bacteria and neutrophils to polymer surfaces as a function of wall shear stress [7,8].

As shown in Figure 3.4, the radial flow chamber contains a Plexiglas housing with two optically flat glass disks, one 75 mm and the other 50 mm in diameter. The 50 mm glass disc is permanently cemented to the top of the chamber and bored with an inlet port in the center. The second disk is made of glass or TCPS. It is removable and can be coated with a polymer test surface before insertion into the chamber. After insertion the chamber is sealed by the presence of O-rings. The 75 mm disk sits at the bottom producing a narrow gap with the top chamber. The gap distance is roughly 300 µm. Fluid enters at the center of the cemented disc, flows over the removable one, and collects in an annular region surrounding the fixed disk. The fluid then exits the chamber via three equidistant ports in the Plexiglas housing of the chamber. The flow field within the radial flow cell can be analytically solved by assuming Newtonian flow between two parallel plates in cylindrical coordinates. The shear rate varies inversely with the radial distance in the chamber. The shear rate at the surface can be shown to be

\[ S = \frac{3Q}{\pi rh^2}, \]

where \( S \) is the shear rate at the surface of the glass disk, \( Q \) is the volumetric flow rate; \( r \) is the radial position, and \( h \) the gap width.

The radial flow chamber was mounted on the motorized stage of a Nikon
inverted microscope. A script in the ImagePro software was composed for the automated control of the stage and the rapid image archiving. Direct observation and counting of the attached cells was possible in real time. The observation field was located at several different positions within each radius as shown in figure 3.4.

A background scan at every scanned flow field was performed with only PBS flowing through the chamber. A EC suspension ($6 \times 10^5$ cells/ml) was introduced into the chamber for the adhesion assay with a continuous field scan over time. The volumetric flow rate was maintained at 3 ml/min using a syringe pump. The resulting shear rate ranged from 0-40 s$^{-1}$. The duration of each independent experiment was about 15 minutes and at least 2 duplicates carried out on each surface.
3.4.2 Cell Staining Procedure for Adhesion on Fibrous Scaffold

For the flow experiment on fibers, cells were lifted and transferred in 15 ml serum free DMEM containing 10μl 5μM cell tracker dye (Molecular Probes). Cells were incubated for 1h at 37ºC and stained blue. Centrifugation at 250g for 5
minutes was subsequently performed. Cells were resuspended into a final concentration of $6 \times 10^5$ cells/ml and injected into the test chamber as described above.

3.5 Statistical Analysis

Statistical software JMP (Cary, NC) was used to statistically compare data and measure differences. One-way analysis of variation (ANOVA) plus Tukey-Kramer analysis were conducted to determine which of the treatments were statistically different. In all tests, a significance value of $\alpha < 0.05$ was used.
4.1 Introduction

After Voorhees made the first vascular graft [1], researchers tried many surface modifications to improve graft blood interactions. A common approach was to mimic the structure of native tissues by seeding endothelial cells (ECs) on the surface, typically with the aid of adhesive proteins or cell binding motifs such as Arg-Gly-Asp (RGD) [2]. However, the binding between cells and the surface is weak. The stripping of ECs after implantation has presented a major challenge for seeded grafts [3].

An alternative is to look for other circulating cells to endothelialize the luminal surface. Recent literature [4] has suggested that a key mediator of the endothelium repair mechanism is the bone marrow-derived mononuclear cell or endothelial progenitor cell (EPC). These cells possess excellent proliferation potential (1,000 population doublings) compared to adult endothelial cells (30 population doublings). They are promising candidates for improving the biocompatibility of vascular grafts [5-7].
In previous studies, materials have been developed which specifically bind to endothelial progenitor cells. Several 12-mer peptide ligands which show binding specific affinity to EPCs were isolated using a phage display technique [8]. The effect of peptides incorporated through chain transfer on EPC binding has been clearly observed in serum-free conditions. However, the cell-specific binding was eliminated when conducting experiments in the presence of serum proteins. Most likely, the peptide-mediated-cell-specific adhesion was masked by the presence binding sites provided by the adsorbed serum proteins. This finding highlighted the need to develop antifouling, protein-repellent base materials to reduce non-specific binding while simultaneously promoting endothelialization.

Methacrylates, which resist biodegradation, are useful for fabricating a small diameter artificial blood vessel. They have been used in the biomedical area for many years, in contact lenses and dental replacement materials [9-11]. In earlier publications, a terpolymer based on hexylmethacrylate and methylmethacrylate and methacrylic acid was described [12]. The cytocompatibility and biostability of the polymer system were illustrated and it was able to be processed into porous scaffolds though electrospinning. PEG is a well-known anti-fouling molecule to limit protein adsorption and non-specific cellular interactions [13]. This chapter describes the incorporation of both the PEG motif and RGD peptides into the polymers to confer biofunctionality even in the presence of serum proteins. This is a prerequisite for usefulness in vivo. Peptides were incorporated either by chain transfer or by coupling with an acrylate-PEG-N-hydroxysuccinimide (NHS) spacer arm. In the case of
chain-transfer, the limited chain end mobility may render some peptides inaccessible to the cells. Peptide incorporation efficiency by chain transfer is also low as characterized by amino acid analysis. Insertion of peptide into polymers can also take place though the N-hydroxysuccinimide (NHS) ester reaction with a primary amine [14], which produces stable amide bonds at the tip of the PEG arm. In the present study, tipped acrylated PEG bound to the RGD peptide was incorporated into the polymer during its synthesis. Since PEG is very hydrophilic, it is expected that peptide tipped PEO chains extend in aqueous solution and provide good ligand accessibility to cell receptors.

4.2 Experimental Methods

In this chapter, polymer were synthesized from hexyl methacrylate (HMA), methyl methacrylate (MMA), poly(ethylene glycol) methacrylate (PEGMA) and CGRGDS peptide. The peptide was incorporated into the polymer system through two ways: a chain transfer reaction and coupling to an acrylate-PEG-N-hydroxysuccinimide (NHS) comonomer. The optimal content of PEG was determined by previous research [15]. According to the feed composition of monomers, polymers were named as H20, H20P15, H20P15RGD etc.. The detailed nomenclature was described in Section 3.1.3 of Chapter 3. The composition of the resulting polymers was determined using NMR, GPC, and amino acid analysis. Polymer hydophilicity and resistance to proteins were determined by water absorption and protein adsorption experiments.
Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from ATCC and cord blood Endothelial Colony Forming Cells (ECFCs) were bought from Lonza. Both types of cells were cultured and experiments carried out on cells retrieved from 4th to 6th generation. HUVECs and cord blood ECFCs were cultured on polymer surfaces in short and long term studies. The short term study lasted for 2 hours while the long term study was carried out over a period of days. At the end of the experiment, cells were stained and imaged. Cell numbers were obtained from the fluorescence generated by a nuclear dye. A difference in number and morphology of endothelial cells was observed depending on the method of peptide incorporation. Beside studies on polymer films, studies on electrospun fiber mats were conducted. Polymer solutions were electrospun into fibrous scaffolds. HUVEC and cord blood ECFC were cultured on them in the same way as on films.

4.3 Polymer Characterization

4.3.1 Polymer Composition and Molecular Weight

Sample NMR spectra are shown in Figure 4.1. Allocating the peaks and analysis of the actual composition were previously reported [15]. Briefly, the chemical shift of the proton A in the HMA is 3.9ppm. Proton B in the terminal methyl group of the MMA is the peak at 3.6ppm. The peak at 3.4ppm represents proton C in PEGMA. With respect to the analysis of H20P15 material, some of the signal from MMA overlaps with proton D from PEGMA.
By studying the spectrum of pure PEG material, we can get the amount of area of PEG that is associated with the area of proton C. The area of protons in MMA is calculated by a simple deduction. In the NMR spectra of H20P15, protons B and D
overlap and were decoupled following the procedures described above.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed Composition (mol% HMA/MMA/PEG)</th>
<th>Polymer Composition (mol% HMA/MMA/PEG)</th>
<th>Mn(KDa)</th>
<th>PDI</th>
<th>Amount of peptide incorporated (nmole peptide /mg polymer)</th>
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<td>5.7±0.5</td>
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<td>25/57/18</td>
<td></td>
<td></td>
<td>3.4±0.2</td>
</tr>
</tbody>
</table>

Table 4.1 Composition and Molecular Weight Averages of Polymers Studied

* The feed compositions of these polymers was 20 mol% HMA, 65 mol% MMA, 14.8 mol% PEG and 0.2 mol% acrylate-PEG-NHS or 0.2 mol% acrylate-PEG- NHSRGD/E. The compositional analysis by NMR did not have the resolution to distinguish between PEG and PEG-NHSRGD/E.

As shown in Table 4.1, the actual content of MMA is lower than the feed composition, which is consistent with a previous report [16]. Even though there is a small deviation in MMA, the overall polymer composition is still similar to that of the feed, indicating the polymerization is close to an ideal copolymerization. The H20 base polymer possesses the highest molecular weight, while the Mw of H20P15RGD is only around 10K. Considering the molecular weight of PEG monomer (500), the 10K molecular weight is fairly low. The high molecular weight of PEG monomer significantly reduces the acrylate reactivity and the introduction of chain transfer reagent further limits the size of the polymer chain. Inserting peptides by NHS chemistry eliminates the lowering of Mw that is caused by chain transfer. The Mw of acrylic-PEG-NHS used in this research is 4 times larger than the PEGMA monomer,
which could have a larger effect on reaction rate and reduce the length of the polymer chain. Since the Mw of H20P15 polymerized from PEGMA is 90K, one can anticipate that the molecular weight of NHS containing polymer is somewhere between the 10K and 90K.

The amount of peptide in each polymer was determined from amino acid analysis performed by Commonwealth Biotech, Inc. (Richmond, VA) and is also shown in Table 4.1. The incorporated peptide by NHS chemistry is larger than the chain transfer mechanism, showing the advantage of the NHS coupling method.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Water Absorption After 30 min (wt%)</th>
<th>Water Absorption After 24 hours (wt%)</th>
<th>Water Absorption At Equilibrium (wt%)</th>
<th>Average Contact Angle (º)</th>
</tr>
</thead>
<tbody>
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</tr>
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</tr>
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<td>171</td>
<td>55.3±2.5</td>
</tr>
<tr>
<td>H20P15NHSRG</td>
<td>49</td>
<td>87</td>
<td>155</td>
<td>54.2±1.7</td>
</tr>
</tbody>
</table>

Table 4.2 Interactions of PEGylated Terpolymer with Water

4.3.2 Interactions of PEGylated Terpolymer with Water

All of the PEG containing polymers are similar to each other with respect to the water interaction (Table 4.2). Intrinsically, the base H20 material is hydrophobic. The incorporation of PEG increases the polymer’s water affinity. At equilibrium, the amount of water absorbed is more than the starting weight. The average contact angles and the amount of water absorbed for the H20 material is statistically different from the polymers containing PEG. Even though some of the materials contain
peptides, the peptide density is too low to affect water absorption and contact angle. One can tell that the dramatic change from H20 to other PEG containing materials is caused by the introduction of the PEG chain.

4.4 Biological Interactions

4.4.1 Protein Adsorption

According to a previous study [12], protein adsorption can undermine the specificity of a peptide which resides on a polymer surface, since adsorbed proteins can be adhesive to many varieties of cells. The incorporation of both PEG and peptide creates a surface with binding affinity to the desired cells but not proteins. Two major serum proteins, fibrinogen (FB) and albumin (BSA) were selected for protein adsorption tests (Figure 4.2). Tissue culture polystyrene (TCPS) was used as a control surface.

The amount of the protein adsorbed on H20 material is higher than that on TCPS. For the adsorption of fibrinogen, the amount on H20 is from 0.8-1.2 µg/cm², which is enough to cover the entire surface area. This is consistent with a previous report [15] and likely due to the hydrophobic nature of the base polymer. Five different PEG containing polymers were included in this comparison. All of them show significant depression of protein deposition. The amount of albumin on PEG containing polymers is seven times lower and that of fibrinogen less than one third lower than that on H20, which indicates that the PEG molecules produce a protein resistant surface.
Figure 4.2 Fibrinogen and Albumin Adsorption on Different Polymers

50 µg/ml solution of fluorescently tagged fibrinogen or albumin interacted with each surface for 40min at 37 ºC

4.4.2 Assessment of cell adhesion

The adhesion of HUVECs on different substrates was evaluated by fluorescence intensity generated by Pico Green® dsDNA nucleic acid stain. HUVECs were detached with EDTA. Cells were resuspended in cell culture media without serum. Figure 4.3 shows the HUVEC adhesion at two hours to surfaces adsorbed with different proteins.
Figure 4.3 HUVEC Adhesion on Different Polymers at 2 hours.

All the surfaces were in contact with protein solution for 1 hour at 37 °C. For the surface H20 and TCPS, the results showed clearly that fibrinogen promotes cell adhesion, while the albumin reduces it. It is expected that the attachment of cells on PEGylated polymers should be dramatically lower than on H20. This is clearly confirmed by the experiment. For all of the materials which contain PEG but not RGD peptide, there is almost no cell adhesion on the surfaces, unless they were treated with FB. The adsorption of FB was reduced by the presence of PEG, but there was enough FB to contribute to the cell binding.

The difference between H20P15, H20P15NHS, H20P15NHSRGD and the PEG functionalized polymers which contain RGD indicates that the surface had both protein resistance and cell binding affinity. The interesting observation is that the number of cells attached seems not to be affected by the protein present on
H20P15NHSRGD surface. Even though it was treated with FB or BSA, the cell density did not deviate too much. This is not surprising since the amount of adsorbed protein was greatly reduced by PEG and the RGD moieties on the polymer surface were able to interact with the transmembrane integrins and help to promote cell adhesion. Unlike the surface incorporated with RGD by NHS chemistry, the RGD motif immobilized via chain transfer shows limited functionality in binding of cells. The density of cell attached on H20P15RGD surface is low but higher than the bare PEG containing surface.

Figure 4.4 summarizes the adhesion and growth of HUVECs and ECFCs on different polymer surfaces through 8 days of incubation. The cell densities on H20P15, H20P15NHS and H20P15NHSRGGE are relatively low at all times. It is not easy for cells to adhere to the PEG containing materials without the support of adhesive peptide. Since H20 and TCPS are intrinsically adhesive to the cells, HUVECs and ECFCs are able to proliferate well on those surfaces. The cell density increases steadily throughout the 8 days. As described above, the functionality of RGD in materials via chain transfer seems to be limited. The cell densities on those surfaces are relatively low. An interesting observation occurs on the H20P15NHSRGD surface. Initially, the number of cells attached to H20P15NHSRGD is almost the same as that on TCPS. Surprisingly, after 8 days, many cells die and detach from the original surface (data not shown). A PEGylated polymer itself is not a cell supporting surface. The introduction of adhesive peptides promotes the initial attachment. From the water adsorption data we know that the amount of adsorbed water at equilibrium is 3 times
higher than that at 30 minutes. After long term contacting with the aqueous solution, the peptide may also be hydrolyzed. One of the remedies might be to reduce the content of PEG which would also affect its non fouling properties. Figure 4.4 also shows us the higher proliferation rate of ECFCs. At 2 hours, there is no significant difference between those two cell lines. However after 8 days, the cell populations of ECFC on H20 and TCPS are more than 3 times larger than that of HUVECs. This indicates the potential of ECFCs to endothelialize grafts.

![Cell density (number/mm²)](image)

**Figure 4.4 HUVEC and ECFC Adhesion on Different Polymers at 2 hours and 8 Days with medium containing 10% serum**

The morphology of HUVECs on the polymer surfaces after 2 hours incubation is shown in Figure 4.5. H20P15 and H20P15NHSRGGE materials not only suppress cell adhesion but also inhibit the spreading of the cells. There is a larger population of cells on H20P15NHSRGD substrate. Cells spread and covered most of the area of the
surface. Cell densities on H20 and TCPS are high, which is in the consistent with the
data in Figure 4.4.

4.4.3 Polymer Processing

PEG and peptides have been widely incorporated in hydrogels for biomedical
applications [17]. Since most hydrogels are thermosets, they can not be processed
post-synthesis [18, 19]. The materials used in this research are produced by the
copolymerization of several acrylate monomers. They keep their thermoplastic
property and can be further fabricated. In this study, we applied electro-spinning
technology to produce polymer scaffolds.

Figure 4.6 shows the SEM image of the electro-spun polymers. Hydrophobic
H20 generates clear and discrete fibers. For the polymers containing PEG, the fibers
coalesce resulting in small pore size. This is possibly also due to the lower molecular
weight and the corresponding lower Tg of the PEG containing polymers.
Figure 4.5 Fluorescence Images of HUVECs Adherent to Polymers After 2 Hours of Culture:

(A) H20P15 (B) H20P15NHSRG (C) H20P15RGD (D) H20P15NHSRGD (E) H20 (F) TCPS
Figure 4.6 Scanning Electron Micrographs of Electrospun Polymers at ×300 Magnification:

(A) H20P15 (B) H20P15NHS (C) H20P15NHSRGE (D) H20P15RGD (E) H20P15NHSRGD

(F) H20
Figure 4.7 HUVEC Adhesion to Electrospun Polymer Scaffolds after 2 Hours of Incubation.

Date are compared with the cell density on films of similar composition.

Figure 4.7 shows the adhesion of HUVECs to the polymer fibers and films after 2 hours of culture. Similar trends are observed comparing the fiber and film adhesion data. Lower cell densities were found on H20P15, H20P15NHS, H20P15NHSRGGE fibers, while there was a higher cell density on H20P15NHSRGD and TCPS scaffolds. The number of cells adhering to the scaffold with only PEG incorporation was significantly greater than that on the analogous film, possibly indicating that an increased number of cells were physically trapped in the fibrous structure [15,20].
4.5 Summary

Polymeric biomaterials were synthesized through the terpolymerization of HMA, MMA, and PEGMA. Incorporation of CGRGDS peptide was achieved by either chain transfer or NHS-RGD coupling chemistry. The succinimide chemistry proved to be a better peptide linking method, since it does not lower polymer molecular weight and also increased the bio-availability of the peptide. There was no difference between mature ECs/HUVECs and ECFCs adhesion at 2 hours of incubation. However over longer time periods ECFCs more readily proliferate. Polymers containing both PEG monomer and peptide motif were found to have excellent resistance to protein adsorption without interfering with the functionality of the peptide. Furthermore, the thermoplastic polymers could be processed into fibrous scaffolds through electrospinning. These materials retained their protein resistance and cell adhesion properties, indicating their potential in vascular graft applications.
CHAPTER 5

THE ADHESION OF HUVECS, CORD BLOOD ECFCS AND HBOECS ON POLYMERS CONTAINING CELL SPECIFIC BINDING PEPTIDES

5.1 Introduction

Synthetic materials can be designed to have mechanical properties useful for constructing vascular grafts, stents, heart valves, and numerous other types of assist devices. However, non-native materials provoke a cascade of pro-coagulant and immune responses that shorten the life of synthetic devices and eventually result in failure [1]. A range of surface treatments has been developed to increase the biocompatibility of synthetic materials with the blood by inhibiting protein adsorption, thrombin formation, and platelet activation [1]. This chapter examines the use a non-fouling approach that promotes the attachment of circulating endothelial progenitor cells with the goal of promoting the development of a native endothelium. The material consists of a synthetic polymer base that incorporates two active species: 1) Polyethylene oxide (PEO), well known for inhibiting the adsorption of platelets and thrombin and minimizing protein binding [2], and 2) novel peptides that anchor only endothelial progenitor cells present in peripheral blood.

Synthetic vascular grafts may be coated with a biological substrate or cellular
layer that mimics the native endothelium as the native endothelium is the only truly blood compatible surface [3]. A promising cellular candidate is the so-called endothelial progenitor cells (EPCs), termed blood outgrowth endothelial cells (HBOECs) after the manner by which they are obtained. The term HBOECs encompasses a heterogeneous population of rare circulating cells that are released from marrow into the blood stream at the instigation of a cascade of tissue damage and distress signals [4]. HBOECs are particularly appropriate as a biocompatible mask because of their native role in vascular repair and high proliferative capacity [5-7]. HBOECs were shown to reendothelialize damaged blood vessels in ischemic organs [8,9] and seem to repair the myocardium following infarction[10,11]. Experimentally, vascular grafts coated with monolayers of HBOECs minimize thrombosis [12]. Because of their appreciable expandability, HBOECs could readily be sourced autologously. Moreover, devices with coatings that confer specific affinity for HBOECs could be directly implanted without the need for in vitro seeding and expansion [12]. A drawback of in vitro culture is that a cell's phenotype is liable to change upon leaving its native environment [13].

Short binding sequences of amino acids have been used to mimic the anchoring of cells to native extracellular matrix, especially RGD and YIGSR peptides that mimic recognition motifs found in fibronectin and laminin, respectively[14-18]. Based on this it is assumed that surfaces that present these or similar binding motifs could recruit cells for attachment in vivo. However, RGD and YIGSR sequences bind a broad array of cells. It is expected that a peptide with affinity for a limited subset of
cells such as HBOECs, the ideal blood contact layer [12], can promote spontaneous endothelialization within the host. In a previous report [18] a phage-display library was screened for peptides with high affinity and selectivity toward HBOECs but not human umbilical vein endothelial cells (HUVECs). These peptides were incorporated into films of methacrylate terpolymer via chain transfer free radical polymerization [19]. Several peptide candidates were identified which anchored HBOECs and supported their function and growth, while these same peptides did not bind HUVECs. The peptides retained their functionality and specificity within the polymer but only in the absence of serum.

Methacrylate polymers, which resist biodegradation and have tunable mechanical properties, have previously been suggested as an alternate for PET and expanded PTFE for fabricating grafts and small diameter vessels [20-22]. They have been used in the biomedical area for many years, in contact lenses and dental replacement materials [20,23-24]. In the previous chapter, a versatile terpolymer was described based on hexylmethacrylate, methylmethacrylate, and methacrylic acid [19]. This polymer can be cast into thin films or electrospun into porous 3D scaffolds. Furthermore, additional molecules can be incorporated into the polymer via the free radical polymerization of a pendant acrylate group. This includes, in particular, molecules that minimize thrombin binding or increase affinity for HBOEC.

In order to minimize the adsorption of serum proteins expected to curtail peptide binding activity, acrylate-modified polyethylene oxide (PEO) was incorporated into the polymer as a monomer in the free radical polymerization. The PEG featured at the
distal end a succinimide molecule to covalently attach the primary amine of the binding peptides. The resulting system consisted of a quadpolymer with tunable mechanical properties, PEG to minimize cell and protein adhesion, and HBOEC binding peptides presented at the end of 2 kDa bristles extending away from the polymer backbone. The activity of the peptides should be enhanced by the elimination of fouling by serum proteins as well as by enhanced mobility of the hydrophilic PEG at the polymer-aqueous boundary.

5.2 Experimental Methods

We previously incorporated RGD and RGE peptides into terpolymer methacrylate films and electrospun scaffolds. In this part several novel peptide identified by the phage display technology have been included in the polymer system. These phage display peptide specifically to adult human blood outgrowth endothelial cells (HBOECs) but not HUVECs. The three 12-mer peptides from the phage display studied in this paper are named after the first three initials of the amino acid sequence: GHM, SYQ, and TPS (Table 2.2).

The other polymers studied were composed of HMA, MMA, PEGMA and CGRGDS/CGRGES peptide. The peptide was incorporated into the polymer system by NHS chemistry. The exact components of the resulting polymers were determined by NMR, GPC, and amino acid analysis. Polymer hydophilicity and resistance to proteins were determined by water absorption and protein adsorption experiments.

Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from ATCC
and cord blood Endothelial Colony Forming Cells (E CFCs) were bought from Lonza. Human blood outgrowth endothelial cells (HBOECs) were derived from human blood donors with the approval of an institutional review board. The surface markers of HBOECs were identified through flow cytometry. The morphology of HBOECs was studied after immunofluorescent staining. All of three types of cells were cultured and proliferated in T flasks before the experiments. For the cell binding assay, HUVEC, cord blood ECFC and HBOECs were cultured on polymer surfaces in short term experiments which lasted for 2 hours. The exact cell number was obtained from the fluorescence generated by the nuclear dye. Cell viability and metabolism were determined using enzymatic activity assay.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed Composition (mol% HMA/MMA/PEG)</th>
<th>Polymer Composition (mol% HMA/MMA/PEG)</th>
<th>Mn(KDa)</th>
<th>PDI</th>
<th>Amount of peptide incorporated (nmole peptide /mg polymer)</th>
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<tbody>
<tr>
<td>H20</td>
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</tbody>
</table>

Table 5.1 Composition and Molecular Weight Averages of Polymers studied

5.3 Polymer Synthesis and Characterization

A schematic of PEG-peptide incorporation into the copolymer was shown in Figure 3.2. The NHS-PEG was first reacted with the amine-terminated peptide. The peptide-PEG was then polymerized with HMA and MMA, leaving the peptides at the tips of bristles. Within aqueous media the hydrophilic PEG chain extend away from
the polymer backbone, allowing good ligand accessibility to cell receptors. The composition of the final polymer composition was controlled by the stoichiometry of the monomers. The actual composition was within +/- 10% of the monomer feed composition. Details of the results from amino acid analysis are also summarized in Table 5.1.

Water absorption measurements (Table 5.2) revealed that the presence of 15 mol% PEG in the polymer increased the equilibrium water uptake five-fold compared to the H2O base polymer that contained neither PEG nor peptides. After thirty minutes PEG-polymer reached nearly 30% of its equilibrium water adsorption and close to 50% saturation after 24 hours.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Water Absorption After 30 min (wt%)</th>
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<th>Water Absorption At Equilibrium (wt%)</th>
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</table>

Table 5.2 Interactions of PEGylated Terpolymer with Water

Both fibrinogen and bovine serum albumin adsorption were significantly reduced by incorporating PEG into the polymer (Figure 4.2). These are major protein constituents of serum and demonstrate the ability of the PEG-polymer to resist protein deposition during blood contact that may mask peptide functionality. With 15 mol%
PEG, fibrinogen adsorption was reduced by half; albumin adsorption was nearly eliminated.

In a previous report [21] it was established that a methacrylate terpolymer had to incorporate at least 2 nmol of RGD peptide per mg of polymer in order to promote HUVEC adhesion. Fussel et al. reported diminished returns with RGD concentrations greater than 5 nmol/mg polymer with a 50% increase in cellular adhesion on going from 2.5 to 5 nmol/mg. In Table 5.2 the H20P15NHS-RGD polymer contained > 5 nmol RGD/mg polymer, the highest peptide incorporation in this study and was the polymer sample which demonstrated the greatest cellular response. H20P15NHS-GHM polymer, for which a modest effect was observed, incorporated ~2 nmol GHM/mg polymer, very close to the threshold limit of the earlier study (Table 5.1). The H20P15NHS-TPS polymer, for which no statistical effect was observed, contained only ~1 nmol/mg polymer. It is likely that the TPS-polymers could have elicited a cellular response at higher levels of peptide incorporation. Further effort should be expended to increase the GHM and TPS peptide content of these polymers.

5.4 Biological Assays

5.4.1 Endothelial Outgrowth Cells from Adult Peripheral Blood

As expected from the EPC literature, after several days in EGM-2 medium a few adherent mononuclear cells from adult peripheral blood began to expand on collagen substrates. The cells formed mature colonies by three weeks. As these cells
approached confluence they exhibited typical cobblestone morphology (Figure 5.1a). They stained positively for Ulex europaeus lectin and took up acetylated low density lipoprotein (Figure 5.1b), which in combination are all markers of endothelial lineage [26]. Flow cytometry of 4th passage adult HBOECs gave 0.3% CD133(+), 1.3% CD34(+), and 23% VEGFR-2(+). Lonza Inc. report that ECFC® express an identical amount of CD34.

Figure 5.1 Images of 4th Passage Adult HBOECs.

Brightfield image of outgrowth cells showing cobblestone morphology (a) and Fluorescence image with positive uptake of green FITC Ulex lectin and red DIL acetylated-LDL (b)

5.4.2 Metabolic Activity

HUVECs and adult HBOECs were incubated with the metabolite indicator CCK-8 to determine if metabolic activity was related to increased cellular adhesion. The metabolic activity of both adult HBOECs and HUVECs was not affected by the presence of peptide nor by the type of peptide in the polymer (Figure 5.2). In all cases,
metabolic activity was several folds greater on tissue culture polystyrene than on the PEG-terpolymer films. Even though same cell densities were seeded on each surface as described in Section 3.3.5, there was little metabolic activity on PEG containing polymers indicating PEG intrinsically does not support the growth of cells.

Figure 5.2 Metabolic Activities of Adult HBOEC (a) and HUVEC (b) on PEG-Polymers and Tissue Culture Polystyrene (TCPS).

The PEG polymer supported lower metabolic activity, regardless of the peptide, compared to TCPS.

### 5.4.3 HBOEC and HUVEC Adhesion to Polymer Films

Figure 5.3 presents cell adhesion to several peptide-containing polymers as measured indirectly via DNA quantification of those cells remaining on the films following a wash step. Cell adhesion was carried out in media containing 10% serum. Tissue culture polystyrene was included as a positive control to ensure that the cells were otherwise healthy. RGE peptide served as a negative peptide control as it is known to have minimal binding affinity. HUVEC adhesion to the peptide-polymer
film was affected only by the presence of RGD (Figure 5.3a), to which it had relatively high affinity. HUVEC reactivity toward the films containing phage display peptides was indistinguishable from the RGE control. Since these peptides were selected for having no activity for HUVEC, this is an expected result. That HUVEC had high affinity for the RGD-film, nearly as high as tissue culture polystyrene, but not the RGE-film as expected.

**Figure 5.3** the General Adhesion Motif RGD Increased Adhesion in All Cell Lines: HUVEC (a), Adult HBOEC (b), and Lonza ECFC (c).

Only the novel peptide GHM selectively increased the adhesion of adult HBOECs.

Adult HBOEC adhesion was significantly enhanced on the GHM peptide containing polymers (Figure 5.3b). The adhesion to GHM-film was half that of RGD-film but specific to HBOECs even in 10% serum medium. This is an advance
over previous studies in which phage-display peptides bound to polymers lost their affinity for HBOECs in the presence of serum proteins. The GHM-peptide had not previously been incorporated into the methacrylate terpolymer and it is encouraging that it has even higher specific affinity for HBOECs than the previous candidates, TPS and SYQ, although those peptides were incorporated at lower concentration.

The other cord-derived cells, Lonza ECFCs (Figure 5.3c), like HUVECs, had high adhesion to the RGD-film but low adhesion to RGE-film and all peptide-films. It is noteworthy that ECFCs had no activity toward peptides that had been selected for their affinity toward HBOECs derived from adult peripheral blood. Lonza ECFCs were derived from umbilical cord blood. The mechanism of peptide activity in this case remains unknown. In light of possible significant phenotypic changes that occur during expansion of primary cells, the precise mode of action is these peptides requires further investigation.

5.4.4 Cell Morphology

In vivo, cells are anchored to the extracellular matrix by receptor-ligand interactions. In the case of a synthetic material, adhesion is initially guided by serum proteins in the medium. In the present case, PEO has minimized the adsorption of serum proteins leaving the phage display peptides as the major contributor to focal adhesion. Structural and signaling proteins within the focal adhesion complexes transmit signals to the cell that govern migration, viability, proliferation, and differentiation (25).
Figure 5.4 Confocal Micrographs (20X) of Adult HBOEC

Cells stained red for F-actin and blue for nuclei, adhering to peptide-polymer films and TCPS. Actin was activated by GHM peptide nearly as much as RGD

Confocal images were taken of HUVEC and adult HBOEC stained for nucleus and filamentous actin after four hours of contact with the peptide polymer films in standard medium. In qualitative terms fewer BOEC (Figure 5.4) were observed on the inactive peptide films (RGE, TPS, and SYQ) compared to the active peptide films (RGD, GHM). HBOEC adhering to RGD- and GHM-polymer films also had more extended F-actin, suggesting these peptides supported a greater number of focal adhesions and active integrin receptors. HBOEC had by far the greatest F-actin extend on tissue culture polystyrene, a surface with high serum protein adsorption. Thus, cell adhesion numbers measured by gross cell count or DNA assay correlated on the
cellular level with F-actin extension.

For HUVEC (Figure 5.5) a significant number of cells could only be found on the RGD-polymer and TCPS. Adsorbed serum proteins were responsible for high F-actin extension in the case of TCPS. The GHM peptide elicited a minimal F-actin extension in HUVEC, corroborating the small number of cells that remained after washing.

Figure 5.5 Confocal Micrographs (20X) of Adult HUVEC

Cells stained red for F-actin and blue for nuclei, adhering to peptide-polymer films and TCPS. Actin was activated only by RGD

5.5 Summary

Methacrylate terpolymers are a promising material for grafts and implants. They feature ease of processing, biostability, and customizable mechanical properties.
Moreover, they have been produced with peptides that confer cell selective adhesion to HBOECs, a cell line that is especially valuable for in-situ endothelialization. A novel methacrylate polymer has been synthesized with both a PEG component to minimize protein adhesion and nonspecific cell binding as well as a peptide binding component. These binding peptides were selected using phage display and express high binding affinity for adult HBOECs. Cast films of the polymer incorporating the GHM peptide significantly increased the contact adhesion and F-actin presentation of adult HBOECs in the presence of serum proteins.

Materials containing GHM and similar peptides may be useful in developing highly proliferative progenitor cell covered devices including vascular grafts. Such an endothelial layer would be inherently non-thrombogenic at the host-device interface.

The problem is that all cell types including HUVECs, ECFCs and HBOECs adhered better to PEG containing polymer films containing adhesive peptides after 2 hours while significant cell detachment occurred after four days. In the future polymer compositions need to be optimized for cell adhesion and cell specificity as well as the support of long term growth.
CHAPTER 6
EFFECT OF PEPTIDE DENSITY AND PEG CONTENT ON CELL ADHESION AND PROLIFERATION

6.1 Introduction

The field of tissue engineering has emerged to address the needs of an increasing number of patients requiring tissue for reconstructive surgery or organs for transplantation [1]. A common theme in tissue engineering is to increase biocompatibility through mimicking the native extracellular matrix (ECM). Many physiological processes involve the reaction between extracellular matrix ligands and cells. It has been shown that the adhesion of cells has a dramatic impact on processes such as wound healing and infection [2, 3]. It is also known that the cell adhesion, migration and signal exchange are mediated by choosing the proper ECM [4, 5].

In many cases, RGD peptides have been immobilized on materials to promote cell adhesion via ligand receptor interactions [7-11]. Several groups have demonstrated enhanced cell adhesion due to the presence of RGD peptides [12, 13]. Indeed, RGD facilitates the binding of many different cell types [6,18,19]. Even though RGD surface modification approach remains a promising strategy, the practical utility of this method is limited by the need for promoting adhesion of a specific cell type. To address this need, Veleva and Cooper have developed materials
which specifically bind to human blood outgrowth endothelial cells (HBOECs) [14]. Several 12-mer peptide ligands which show binding specific affinity to EPCs were isolated using a phage display technique [15]. The effect of cell specific binding peptides incorporated through chain transfer was clearly observed in serum-free conditions. However, in the presence of serum proteins, the cell-specific binding was eliminated [14].

In order to minimize the adsorption of proteins, acrylate-modified polyethylene glycol (PEG) was incorporated into the polymer as a monomer in the free radical polymerization. The cell specific binding was successfully achieved with the presence of serum due to the incorporation of PEG as the peptide linker [16,17]. Although both PEG and peptide play an important role with respect to the initial adhesion and proliferation of cells, the effects of PEG and adhesive peptides work against with each other. PEG prevents adhesive protein deposition and thus attenuates cellular attachment while cell binding peptides promotes cell adhesion. In previous studies, cell proliferation was rapid initially, but significant cell loss was observed after 2 days incubation [16]. It has been found that 5% incorporation of PEG is sufficient to prevent the deposition of proteins while significantly supporting the growth of cells for 15 days [17]. This Chapter is an exploration of PEG and RGD peptide concentrations in a search for the optimal promotion of cell proliferation on a non-fouling surface.

In this Chapter the initial and long term adhesion of cord blood endothelial colony forming cells (ECFCs) and human umbilical vein endothelial cells (HUVECs)
to different polymer surfaces in static conditions is described. The influence of adhesive peptide content was also explored over a range of shear rates. In general, cord blood ECFCs exhibited greater adhesion under all conditions.

### 6.2 Experimental Methods

The base polymer in this chapter was composed of HMA, MMA, PEGMA and CGRGDS peptide. The peptide was incorporated into the polymer system by coupling to an acrylic-PEG-NHS comonomer. Three different peptide content were used: 0mg peptide/g polymer, 30mg peptide/g polymer and 60mg peptide/g polymer. Three different PEGMA content were also synthesized: 0mol%, 7mol% and 15mol%. For all polymers, 0.2mol% of acrylic-PEG-NHS was used so that all polymers had the same composition except for RGD and PEGMA content. The composition of the polymers was determined by NMR, GPC, and amino acid analysis. Polymer hydophilicity was determined by water absorption and contact angle experiments.

Two types of cells were used: HUVECs and Lonza ECFCs. Both of cell types were cultured and proliferated in flasks until the 4-6th generation. Then HUVECs and cord blood ECFCs were cultured on polymer surfaces in short term and long term studies. The short term study was carried out for 2 hours while the long term study was on the order of several days. At the end of the experiment, cells were stained and imaged using a fluorescence microscope. Cell number was obtained from the fluorescence generated by the dye as described in Section 3.3.2. A difference in number and morphology of endothelial cells was observed depending on the polymer
compositions.

Beside cell adhesion experiments carried out under static conditions, studies under flow were conducted. A radial flow chamber was used to study EC adhesion to surfaces in the presence of an applied shear stress.

6.3 Polymer Characterization

The schematic of PEG-peptide incorporation into the copolymer was shown in Figure 3.2. The NHS-PEG was first reacted with the amine-terminated RGD. The peptide-PEG was then polymerized with HMA and MMA, leaving the peptides at the tips of the PEG chains. Within aqueous media the hydrophilic PEG chain are expected to extend away from the polymer backbone, allowing the peptides to provide good ligand accessibility to cell receptors.

A summary of the methacrylate copolymer compositions studied is shown in Table 6.1. Like other chapters, the polymer is referred by the mole percent of the monomer and the composition MMA is omitted in the terminology. The last letter 'H' indicates the 60mg/g RGD incorporation while letter 'L' indicates 15mg/g. H20 refers to a base material synthesized from 20/80 mol % HMA/MMA. H20P15NHSRGD refers to a polymer copolymerized from 20/65/14.8/0.2 mol % HMA/MMA/PEGMA/acrylate-PEG-RGD. H20P15NHS refers to a material copolymerized from 20/79.8/0/0.2 mol% HMA/MMA/PEGMA/acrylate-PEG-NHS. There was no RGD incorporation into this polymer. As shown in the table, the actual content of MMA is lower than the feed composition, which is consistent with previous reports [20,21].
The overall polymer composition is still similar to that of the feed, indicating ideal copolymerization conditions.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed Composition (mol% HMA/MMA/PEG)</th>
<th>Polymer Composition (mol% HMA/MMA/PEG)</th>
<th>Mn(KDa)</th>
<th>PDI</th>
<th>Amount of peptide incorporated (nmole peptide /mg polymer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H20</td>
<td>20/80/0</td>
<td>23/77</td>
<td>203596</td>
<td>2.06</td>
<td></td>
</tr>
<tr>
<td>H20P15NHSRGD-H</td>
<td>20/65/15</td>
<td>21/63/16</td>
<td></td>
<td></td>
<td>7.61±3.6</td>
</tr>
<tr>
<td>H20P15NHSRGD-L</td>
<td>20/65/15</td>
<td>23/58/19</td>
<td></td>
<td></td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>H20P15NHS</td>
<td>20/65/15</td>
<td>21/62/17</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>H20P7NHSRGD-H</td>
<td>20/73/7</td>
<td>25/65/10</td>
<td></td>
<td></td>
<td>6.97±0.2</td>
</tr>
<tr>
<td>H20P7NHSRGD-L</td>
<td>20/73/7</td>
<td>24/65/11</td>
<td></td>
<td></td>
<td>0.91±0.2</td>
</tr>
<tr>
<td>H20P7NHS</td>
<td>20/73/7</td>
<td>23/67/10</td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>H20P0NHSRGD-H</td>
<td>20/80/0</td>
<td>21/77/2</td>
<td></td>
<td></td>
<td>2.9±1.7</td>
</tr>
<tr>
<td>H20P0NHSRGD-L</td>
<td>20/80/0</td>
<td>23/75/2</td>
<td></td>
<td></td>
<td>1.3±1.1</td>
</tr>
<tr>
<td>H20P0NHS</td>
<td>20/80/0</td>
<td>21/78/1</td>
<td></td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Table 6.1 Composition and Molecular Weight Averages of Polymers studied

The amount of peptide incorporated was determined from amino acid analysis performed by Commonwealth Biotech, Inc. (Richmond, VA) and shown in Table 6.1. In a previous report [22], it was established that a methacrylate terpolymer had to contain at least 2 nmol of RGD peptide per mg of polymer in order to promote HUVEC adhesion. In this research, most of the surfaces contain peptide concentrations greater than 2nmol/mg except H20P7NHSRGD-L and H20P0NHSRGD-L, which are close to 1nmol/mg.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Water Absorption After 30 min (wt%)</th>
<th>Water Absorption After 24 hours (wt%)</th>
<th>Water Absorption At Equilibrium (wt%)</th>
<th>Average Contact Angle (º)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H20</td>
<td>2</td>
<td>2</td>
<td>28</td>
<td>80.1±2.1</td>
</tr>
<tr>
<td>H20P15NHSRGD-H</td>
<td>39</td>
<td>65</td>
<td>152</td>
<td>51.0±2.8</td>
</tr>
<tr>
<td>H20P15NHSRGD-L</td>
<td>42</td>
<td>74</td>
<td>171</td>
<td>55.3±2.5</td>
</tr>
<tr>
<td>H20P15NHS</td>
<td>40</td>
<td>78</td>
<td>169</td>
<td>54.4±3.9</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>----------------</td>
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<td>----</td>
<td>-----------</td>
</tr>
<tr>
<td>H20P7NHSRGD-H</td>
<td>22</td>
<td>42</td>
<td>53</td>
<td>69.7±2.3</td>
</tr>
<tr>
<td>H20P7NHSRGD-L</td>
<td>19</td>
<td>50</td>
<td>51</td>
<td>71.0±2.1</td>
</tr>
<tr>
<td>H20P7NHS</td>
<td>19</td>
<td>39</td>
<td>49</td>
<td>70.2±2.6</td>
</tr>
<tr>
<td>H20P0NHSRGD-H</td>
<td>0.5</td>
<td>0.6</td>
<td>24</td>
<td>80.5±3.5</td>
</tr>
<tr>
<td>H20P0NHSRGD-L</td>
<td>5</td>
<td>10</td>
<td>30</td>
<td>78.4±5.4</td>
</tr>
<tr>
<td>H20P0NHS</td>
<td>2</td>
<td>8</td>
<td>19</td>
<td>82.1±0.3</td>
</tr>
</tbody>
</table>

Table 6.2 Interactions of PEGylated Terpolymer with Water

Water absorption measurements (Table 6.2) show that an increase in PEG content in the polymer increased the hydrophilicity as expected. The equilibrium water adsorption of P15 material is five times higher than the P0 surface. After thirty minutes PEG-polymers reached nearly 30% saturation and close to 50% saturation in 24 hours. P7 samples swell 50% after 2 weeks roughly one third of the water absorption of the P15 materials.

6.4 The Adhesion of Endothelial Cells under the Static Conditions

6.4.1 The adhesion of Cord Blood ECFC on Polymer Surfaces

The adhesion of ECs on different substrates was evaluated by fluorescence intensity generated by Pico Green® dsDNA nucleic acid stain. Both ECFCs and HUVECs were detached with EDTA. Cells were resuspended in cell culture media without serum. Figure 6.1 shows the ECFC adhesion on polymers with high RGD content. Incubation time was from 2 hours to 8 days.
Figure 6.1 ECFC Adhesion on Polymers with High RGD Content

The difference between P15, P7 and P0 is dramatic. When the RGD content is high, only the P7 material and the positive control showed the sustainable proliferation of cells. From the amino acid analysis, all of the polymers with high RGD seeding content have the actual RGD composition higher than the critical cell promotion value 2nmol/mg. Without the effect of PEG, they are capable of supporting cell growth. Cell densities on surfaces were similar to each other up to 4th day.

A PEGylated polymer itself is not a cell supporting surface. From Table 6.2, the P15 material will adsorb more than 150% water after a week. This dramatically changes the physical and chemical properties of the material which may directly impact the peptide accessibility. From Table 6.1, the P0 material still contains 1 or 2 mol% PEG and its peptide density is 2 or 3 times lower than others. Although the P0
material is generally hydrophobic and adhesive to proteins, a few cells died and detached from the surface. The most interesting surface is the P7 material. The growth rate of cells was high approaching that of the TCPS positive control. At equilibrium, the P7 material absorbs 50% water and supported cell growth.

![Figure 6.2 ECFC Adhesion on Polymers with Low RGD Content](image)

When the RGD content is low, ECFCs did not proliferate on the hydrophilic P and P15 surfaces. Figure 6.2 summarizes the adhesion and growth of ECFCs on different polymer surfaces with low RGD content through 8 days of incubation. At 2 days, all cell densities were similar. By the 4th day, cells began to slough off on P15 and P7 surfaces. The cell densities become fairly low by the 8th day. Since P0 and TCPS are intrinsically adhesive to the cells, ECFCs were able to proliferate on those
surfaces.

6.4.2 Cord Blood ECFC Morphology

Fluorescence images were taken of nuclear and filamentous actin stained cord blood ECFCs after four hours of contact with the peptide polymer films in standard medium. Figure 6.3 A, B and C shows the ECFC adhesion on P7 surface at low RGD content. The cell density increased initially and then decreased. D,E and F shows he ECFCs adhesion on P7 surface when the RGD content is high. ECFCs had more extended F-actin, suggesting these peptides supported a greater number of focal adhesions and active integrin receptors. The P15 surface with low peptide incorporation elicited minimal F-actin extension in ECFCs, corroborating the small number of cells that remained after washing (Figure 6.3 G, H and I). ECFCs had by far the greatest F-actin extension on tissue culture polystyrene (Figure 6.3 J, K and L), a surface with high serum protein adsorption. Thus, cell adhesion numbers measured by gross cell count or DNA assay were correlated on the cellular level with F-actin extension.
Figure 6.3 Fluorescence Micrographs (20X) of Cord Blood ECFC

Cells stained red for F-actin and blue for nuclei, adhering to peptide-polymer films and TCPS

A) 1 day on H20P7NHSRGD-L material, B) 2 day, C) 8 day

D) 1 day on H20P7NHSRGD-H material, B) 2 day, C) 8 day

G) 1 day on H20P15NHSRGD-L material, B) 2 day, C) 8 day

J) 1 day on TCPS, B) 2 day, C) 8 day
6.4.3 The Adhesion of HUVEC on Polymer Surfaces

Figure 6.4 and 6.5 shows us the lower proliferation rate of HUVECs when RGD content is high and low respectively. There is no surface that sustains the proliferation of HUVECs except TCPS while the P7 surface supported the long term growth of ECFCs but not that for HUVECs. Within 2 days, there is no significant difference between ECFCs and HUVECs regarding cellular growth. However after 2 days, the cell populations of ECFCs on all surfaces are larger than those of HUVECs, especially on TCPS. This indicates a greater proliferation potential of ECFCs.
6.6 The Adhesion of Cord Blood ECFCs under Flow

The comparison of ECFC cell attachment on different polymer surfaces at different shear rates after 10 minutes is shown in Figure 6.6. For all surfaces, the number of adhered cells increased as the shear rate decreased. All of the surfaces were almost saturated with cells at 5 s\(^{-1}\) shear rate after 10 minutes. Minimal cell adhesion was found at the shear rate of 20 s\(^{-1}\) and 30 s\(^{-1}\). An interesting result is that cell adhesion on H20P15NHSRGD-L and H20P7NHSRGD-L are higher than that on TCPS. In all of the flow experiments, the total flow time was only about 15 minutes. Since there was no difference at 2 hours under the static conditions, it is not surprising that those surfaces with low RGD content are comparable to TCPS at 10 minutes.
Figure 6.6 Dynamic Adhesion of Cord Blood ECFCs to Polymers and TCPS after 10 Minutes

(A) Adhesion of ECFCs and different polymer films

(B) Bright field image of ECFC under flow. The polymer was H20P15NHSRGD-H and the shear rate was 5s⁻¹
6.6 Summary

A series of polymeric biomaterials were synthesized through the terpolymerization of HMA, MMA, and PEGMA. Incorporation of CGRGDS peptide was achieved by NHS-RGD coupling chemistry. RGD content ranged from 60mg/g, 30mg/g to 0. PEG content ranged from 15mol%, 7mol% to 0. There is no difference between mature ECs and ECFCs adhesion at 2 hours of incubation. However over longer time periods ECFCs more readily proliferate. Polymers containing 7 mol% of PEG and the highest content of RGD supported sustainable growth of ECFCs. In contrast, HUVECs were poorly attached. Cell losses were observed on all surfaces. All RGD containing surfaces promoted the attachment of ECFCs under flow.
CHAPTER 7
THE ADHESION OF HUVEC, CORD BLOOD ECFC AND HBOEC ON RGD LINKED POLYMERS UNDER FLOW CONDITIONS

7.1 Introduction

The luminal surface of human blood vessel is covered with a monolayer of endothelial cells (ECs). It is known that the EC layer can significantly prevent blood protein deposition and the formation of thrombus, which makes the adhesion of ECs to artificial surfaces desirable in synthetic vascular grafts [1-3]. There has been considerable research attempting to endothelialize material surfaces, such as EC seeding, employing EC specific ligands and pre-treating surfaces with adhesive proteins, etc [4-7]. However, those methods require isolation of the host’s endothelial cells and the pre-culturing of such cells on the material surface before implantation. Furthermore, such a seeded endothelium is often undergoes damage during implantation.

Recently a particular group of cells known as Endothelial Progenitor Cells (EPCs) have shown to have great clinical potential. These are bone-marrow derived cells that have the capacity to differentiate into endothelial cells [8]. At least two distinct populations have been discovered: early outgrowth colony-forming cells and
late outgrowth colony forming cells (ECFCs) [9]. Late outgrowth ECFCs are more endothelial cell like and they can be harvested from both cord and peripheral blood. Cells from peripheral blood are also known as human blood outgrowth endothelial cells (HBOECs) [10]. ECFCs possess higher proliferation potential and produce fewer growth factors than early outgrowth cells, which is less likely to promote neointimal hyperplasia [11]. These findings suggest the use of ECFCs as a suitable source of cells for constructing functional tissue engineered blood vessel replacements. However, those advantages are offset by the current complex and time consuming isolation procedures especially for HBOECs. Many animal species can readily endothelialize vascular grafts from adjacent tissue. An alternative strategy for human clinical applications is to develop materials that self endothelialize by harvesting ECFCs from the circulation.

Previous studies described the synthesis of hexylmethacrylate (HMA), methylmethacrylate (MMA), and methacrylic acid (MAA) terpolymers to produce non cyto-toxic and bio-stable surfaces for the growth and expansion of mature endothelial cells (HUVECs) [12-14]. Here, we wish to create a material tuned to capture circulating ECFCs that has the potential to generate a confluent functioning endothelium \textit{in vivo}. Endothelial cells are typically exposed to blood flow induced shear forces under physiological conditions. However the effect of flow on the initial adhesion of endothelial cells to surfaces has received little study. Since the response of ECs may differ substantially from those under static conditions [15-17], a radial flow chamber was used to study EC adhesion to surfaces under flow. Peptide
incorporation was achieved by coupling with an acrylate-PEG-N-hydroxysuccinimide (NHS) spacer arm. The primary amine in the peptide can react with N-hydroxysuccinimide (NHS) ester producing stable amide bonds at the tip of the PEG arm [18]. When exposed to hydrophilic media, the RGD group is expected to provide good ligand accessibility to cell receptors.

In this Chapter, the initial adhesion of HUVECs, cord blood ECFCs and HBOECs to surfaces under well defined flow conditions is examined. The influence of adhesive peptides was explored over a range of shear rates. Both cord blood ECFCs and HBOECs exhibited greater adhesion under flow especially on RGD containing polymers. In general, EC adhesion was highly shear rate dependent and decreased markedly at higher shear rates.

7.2 Experimental Methods

Several non fouling peptide grafted polymers were synthesized as the substrates studied in this Chapter. The polymer was composed of HMA, MMA, PEGMA and CGRGDS peptide. The peptide was incorporated into the polymer system by NHS chemistry. The composition of the resulting polymers was determined by NMR, GPC, and amino acid analysis. Polymer hydophilicity were determined by water absorption and contact angle experiments. The surface of a variable shear-rate device was either coated with a polymer film or covered by synthetic fibers. Spin-coating was applied to produce smooth polymer films while fibrous scaffolds were generated by electro-spinning.
HUVECs were bought from ATCC while ECFCs were purchased from Lonza. HBOECs were derived from human blood samples following Ohio State University protocols. The cells were cultured and proliferated in flasks and generations 4-6 were used in the experiments. The adhesions of HUVECs, cord blood ECFCs and HBOECs under shear stress were determined using a radial flow chamber. Programmed software controlled the stage of a video-microscope for the rapid capturing of images. Polymer fibers were generated by electro-spinning and mounted in the flow cell. In order to visualize cells on polymer fibers, cell tracker dye was applied to stain the cells alive.

7.3 Polymer Characterization

The NMR spectra of the two functionalized polymers look similar to each other since the amount of peptide is below the detection range of the NMR technology used. Figure 4.1 shows NMR spectra of the polymer without peptide incorporation. Allocating the peaks and analysis of the actual composition were followed as previously reported [19]. Briefly, the chemical shift of the proton A in the HMA is 3.9ppm. Proton B in the terminal methyl group of the MMA is the peak at 3.6ppm. The peak at 3.4ppm represents proton C in PEGMA. With respect to the analysis of the H20P15 material, some of the signal from MMA overlaps with proton D from PEGMA. By studying the spectrum of pure PEG, we can get the amount of area of PEG that is associated with the area of proton C. The area of protons in MMA is calculated by simple deduction.
Two peptides were used in this research, RGD and RGE. Details of peptide incorporation along with the summary of the methacrylate copolymer compositions studied were shown in Table 4.2. The actual content of MMA is lower than the feed composition, which is consistent with a previous report [19]. Even though there is a small deviation in MMA, the overall polymer composition is similar to that of the starting monomer composition.

The base H20 material is hydrophobic and processes a significantly larger contact angle than other materials (Table 4.2). After incorporation of 15 mol% PEG units, the water contact angle decreased by about 30 degrees. The two PEG containing polymers are similar to each other with respect to the average contact angles.

### 7.4 Biological Interactions

#### 7.4.1 Adhesion Pattern and the Effect of Lifting Conditions

Dickinson and Cooper [20] observed that the number of adherent bacteria on a surface increased linearly with time at a given shear rate in the radial flow apparatus. At low flow rates on TCPS, cord blood ECFCs also adhered linearly with time, but at higher shear rates, a steady state was reached at lower cell densities illustrating shear dependent cellular adhesion as observed in Figure 7.1. At 30 s⁻¹ shear rate, cellular adhesion on TCPS was minimal. As the shear rate decreases, more and more cells attached in the observation area. At 5 s⁻¹ shear rate, nearly 70% of the surface was covered by cells after 10 minutes. By counting the number of attached cells at 10 minutes, one can compare the degree of cell adhesion under different conditions.
Figure 7.1 Real Time Shear Dependent Cord Blood ECFCs Adhesion to TCPS

Figure 7.2 summarizes the effect of shear rate on cord blood ECFCs adhesion on TCPS using three cell lifting agents. It was observed even in the absence of serum proteins that the cell adhesion was highest on EDTA lifted cells, somewhat lower for 0.025% trypsin /EDTA lifted cells and lowest on 0.25% trypsin /EDTA lifted cells. At 5s-1 shear rate, the difference between lifting conditions is not significant. However at higher shear rates, trypsin lifted cells had much lower adhesion. In the subsequent experiments, all the cells were detached by trypsin free EDTA.
7.4.2 Dynamic Adhesion of HUVECs and Cord Blood ECFCs to Unfunctionalized Polymer Films.

A comparison of HUVEC and cord blood ECFC cell attachment at 10 minutes on TCPS is shown in Figure 7.3. The number of adherent HUVECs is less than that of cord blood ECFCs for the range of shear rates examined (5 to 30s⁻¹). At higher shear rates, the adhesion of cord blood ECFCs is higher by a factor of 3 to 4 compared with that of HUVECs. Figure 7.3 also shows that cord blood ECFCs are less sensitive to shear rate when adhering to H2O. There are more cord blood ECFCs on hydrophobic H2O surface especially at higher shear rates.
Figure 7.3 Dynamic Adhesions of HUVECs and Cord Blood ECFCs to TCPS and H20 after 10 Minutes

7.4.3 Dynamic Adhesions of HUVECs and ECFCs to Functionalized Polymer Films

Figure 7.4 shows the dynamic adhesion of HUVECs to the functionalized polymer surfaces at 10 minutes. The 30s-1 shear rate is too high for any cells to adhere on all the surfaces examined. At lower shear rates, there are almost no cells on H20P15NHSRGGE substrate indicating that the presence of PEG significantly reduced the degree of cell adhesion. At all shear rates, the cell density on TCPS, the positive control, is the highest. H20P15NHSRGD showed progressively more cell adhesion as the shear rate was lowered.
The comparison of HUVECs, ECFCs and HBOECs cell attachment at 10 minutes on functionalized polymer films is shown in Figure 7.5. For all three cell types, the number of adhered cells increased as the flow rate decreased. Minimal cell adhesion was found on the H20P15NHSRG surface. An interesting result is that the cord blood ECFCs adhesion was much higher than that of HUVECs over the entire shear rate range examined. The H20P15NHSRG surface was almost saturated with cells at 5 s⁻¹ shear rate after 10 minutes. The cell density of HBOECs was similar with that of cord blood ECFCs at low shear rates. A clear difference was observed between those two types of ECFCs at 15 s⁻¹. HBOECs are less adhesive compared with cord blood cells but still more adhesive than HUVECs.
Figure 7.5 Dynamic Adhesions of HUVECs, Cord Blood ECFCs and HBOECs to Functionalized Polymer Films after 10 Minutes

(A) Adhesion of HUVECs and both types of ECFCs on H20P15NHSRGD films

(B) Adhesion of HUVECs and both types of ECFCs on H20P15NHSRGE films
7.4.4 Dynamic adhesion of HUVECs and ECFCs to functionalized polymer fibers

Figure 7.6 Scanning Electron Micrographs of Polymer Fibers and Fluorescence Microscope Images of Cells Adherent on Fibers

(A) SEM of electrospun H20P15NHSRGE fibers at ×400 magnification

(B) Cord blood ECFCs on H20P15NHSRGE polymer fibers after 10min using fluorescence microscopy

Figure 7.6A shows the SEM image of the electro-spun H20P15NHSRGE
polymer. The materials used in this research are produced by the copolymerization of several acrylate monomers including the hydrophilic PEG chain. The PEG containing fibers seem to coalesce resulting in small pore size. All the fibers containing PEG with and without peptide look similar to each other. They all have the sponge like structure as illustrated in Figure 7.6A. Figure 7.6B shows the adherent cells glowing blue on the polymer fibers at $5 \text{s}^{-1}$ shear rate after 10 minutes.

![Figure 7.7 Dynamic Adhesions of HUVECs, Cord Blood ECFCs and HBOECs to Functionalized Polymer Fibers after 10 Minutes at $5 \text{s}^{-1}$*.](image)

*Cells tested on fibers were stained with cell tracker dye while cells tested on films were not.*

A comparison of HUVEC, cord blood ECFC and peripheral blood ECFC cell attachment after 10 minutes at the lowest shear rate on fibers is shown in Figure 7.7. Since the fibers are opaque using bright field microscopy in the flow cell, the cells were stained blue in order to count them using fluorescent illumination. Similar trends
were observed to that on films. The RGD peptide promoted the adhesion of all cell types and both ECFCs exhibited greater adhesion than HUVECs. There are no significant differences between cord blood ECFCs and HBOECs on fibers and films at low shear rate. The number of attached cells was lower on fibers than what was found on films.

7.5 Factors that Affect the Adhesion of Cells under Flow

ECFCs are a promising cell type in the field of tissue engineering as a means of improving biocompatibility of vascular grafts. The effectiveness of using ECFCs is limited by the time and complex procedures required to expand them in vitro. An alternative is to capture ECFCs directly from blood. A radial flow chamber was used to study peptide linked polymers to characterizing the real time adhesion of cells under flow. The results show that the degree of EC adhesion on surfaces under flow depends on material hydrophilicity, peptide density, cell lifting conditions, shear rate, and the type of cell.

The hydrophilicity of a material is determined by its chemistry, mainly the PEG motif in this research. Even though some of the materials are functionalized by peptide, the peptide density is too low to affect hydrophilicity. The base polymer H2O without any PEO is hydrophobic which is demonstrated by the water contact angle data in Table 4.2. A hydrophobic surface is usually adhesive for proteins and cells due to the entropy gain [22] in the deposition process, which might be the reason that cord blood ECFCs had higher adhesion on H2O compared to TCPS as illustrated in Figure
7.3. One can tell that the dramatic increase of surface hydrophilicity from H2O to other materials is caused by the introduction of PEG. Since PEG is a well known anti-fouling molecule, both HUVEC and ECFC adhesion was minimal even at the lowest shear rate examined.

The PEG containing polymers are intrinsically passive for cellular attachment. On those surfaces, peptide plays a major role in cell capture. In previous experiments on HUVECs, 2nmol/mg RGD promoted robust HUVEC adhesion [14]. The peptide density in present research is comparable and the results are consistent with these earlier observations. As RGE is non adhesive, the PEG-RGE surface was non adhesive to both types of cells at all shear rates. Compared to the PEG-RGE peptide containing surfaces, much higher HUVEC and ECFC adhesion was observed on the PEG-RGD surface, especially at low shear rates.

An important and often neglected factor in studies of cell adhesion is the cell lifting conditions. The degree of cell adhesion was significantly reduced if the cells were lifted by trypsin. In this study, the effect became more obvious with cells exposed to higher shear rates. While these changes in cell adhesion may be attributed to damage of transmembrane proteins due to trypsin digestion, it was interesting to see an effect on adhesion even though there was minimal adsorbed protein on the polymers [23].

Another important factor that affects cell adhesion is the shear rate. Relatively more cells stayed on the surface at low flow rate as shown in Figures 7.4 and 7.5. Surfaces exposed to the highest shear rates barely support cell adhesion. The ligand
receptor reaction requires time to take place [24], so slower flow provides more time for cells to interact to peptides. Also higher shear rates were observed to remove cells due to the higher shear stresses on the cells. Both factors can rationalize low cell adhesion and the plateau observed at higher shear rates. At lower flow rates the number of attached cells increased linearly with time. The negative relationship between shear rate and the number of cells adherent indicates that convection dominates the process of cell transport. If cell adhesion was controlled by diffusion, more cells should adhere at higher shear rates.

This study also suggests an advantage of using ECFCs as an alternative cell source to endothelialize biomaterials for tissue engineering. The adhesion of HUVECs, cord blood ECFCs and HBOECs was studied and compared to that of control polymer, TCPS, RGD films and RGD fibers. In Figure 7.5, the number of adherent ECFCs on RGD films was three to five fold higher than that of HUVECs. Both cord blood ECFCs and HBOECs are smaller in size and it may be that the forces required to stop them are significantly reduced. At low flow rates, most of the RGD surface can be endothelialized with ECFCs within 10 minutes. The difference between cord blood ECFC and HBOEC was minimal at low flow rates. Cord blood ECFC showed superior adhesion ability at higher shear rates. Compared to HUVECs, the procedure required to harvest cord blood ECFCs or HBOECs is and tedious and can take weeks of culture. HBOECs may express more mature EC phenotype [25] and become less robust in adhering compared with cord blood ECFCs. However considering their long life time and fast expansion rate, both cord blood ECFCs and
HBOECs are promising cell sources to create an endothelial layer under flow conditions. For cell adhesion on fibers, fewer cells attached on the fibrous mats. In a previous report, more cells were retained on the fibrous mats under static conditions since some of the cells were physically trapped [26]. However in the current work, the small pore size, flow and the turbulence formed near the irregular surface may have decreased cell adhesion. In addition, for experiments using fibers, all of the cells were stained by the cell tracker dye which might affect the adhesion characteristics of the cells.

Future work will employ phage display selected peptides directed to HBOECs [27] with the aim of capturing circulating progenitor cells under flow.

7.6 Summary

The Chapter describes a conceptual framework for a system that can capture ECFCs in vivo. Surface properties and shear rate influence the degree of cell adhesion and highlighted the superior adhesion ability of both types of ECFCs compared to HUVECs. Surface hydrophobicity, the presence of PEG moieties and RGD peptide, can significantly affect the efficiency of cell adhesion. The adhesion of cells to surfaces is the result of competition between cell-surface interactions and hydrodynamic forces. RGD promotes cell adhesion while high shear rates reduce the number of attached cells. The cell density of both types of ECFCs was higher than that of HUVEC on all of the surfaces within the entire shear rate range examined. Similar trends were observed on electrospun fibers of the same materials.
CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

The work presented in this dissertation describes the development of a non-fouling peptide grafted polymer for small diameter vascular graft applications. The polymer was composed of hexyl methacrylate, methyl methacrylate, poly (ethylene glycol) methacrylate (PEGMA), acrylate -PEG-N-hydroxy-succinimide (NHS) and various cell binding peptides. Methacrylate terpolymers are a promising material for grafts and implants. They feature ease of processing, biostability, and customizable mechanical properties. The inclusion of PEG renders the polymers hydrophobic. The amount of protein adsorbed on the polymers without PEG was higher than that on tissue culture polystyrene (TCPS). However the degree of cell spreading and the rate of cell proliferation on TCPS positive control was greater than the control methacrylates suggesting bio-active peptide should be included for the improvement of endothelialization as well as bio inert molecules (PEG) to reduce non specific protein adhesion.

Incorporation of peptides was achieved by either by a chain transfer reaction or by coupling to an acrylate-PEG-N-hydroxysuccinimide comonomer. Polymers containing both PEG monomer and peptides were found to have excellent resistance to protein adsorption without losing the functionality of the peptide. Human Umbilical
Vein Endothelial Cells (HUVECs) and Endothelial Colony Forming Cells (ECFCs) were cultured on these surfaces in short term and long term studies. The succinimide chemistry proved to be a better peptide linking method than chain transfer chemistry, since it does not lower polymer molecular weight and also increased the availability of the peptide. Both cell types adhered better to polymer films containing NHS coupled RGD peptide at 2 hours even in the presence of albumin but over longer time periods ECFCs more readily proliferate more readily. It appears that Endothelial Progenitor Cells (EPCs) such as cord blood ECFCs and Human Blood Outgrowth Endothelial Cells (HBOECs) are better at endothelializing polymer surfaces than HUVECs.

A set of peptides from a phage display library binding specifically to adult human blood outgrowth endothelial cells (HBOECs) but not HUVECs were bound to the PEG-NHS monomers and incorporated into polymers. Previously the functionality of these peptides was undermined by the binding sites of non-specifically adsorbed serum proteins. The PEG-NHS peptides polymerize at one end into the polymer backbone and display their succinimide bound peptides at the distal end. Adding PEG to the methacrylate terpolymer curtailed nonspecific protein adsorption and promoted the accessibility of the peptide and helped to prevent non-specific protein adsorption. RGD based peptides as well as the peptides suggested by the phage display technology for HBOECs were tested. Cast films of polymers incorporating the 12-mer binding peptide GHMDMSPHAVID-GGGS (GHM) significantly increased the contact adhesion and F-actin presentation of HBOECs in the presence of serum.
proteins. Materials based on this and similar peptides may be useful in developing progenitor cell capturing to create a native and functional endothelium.

Although cell binding peptides play an important role in the initial adhesion and proliferation of cells, the effects of PEG and adhesive peptides work against each other. PEG containing polymers minimized protein deposition and thus attenuated cellular attachment while cell binding peptides promote cell adhesion. For both ECFCs and HUVECs, significant cell detachment occurred after four days. A series of polymeric biomaterials with different PEG content were synthesized through the polymerization of HMA, MMA, acrylate-PEG-NHS and PEGMA. Incorporation of CGRGDS peptide was achieved by NHS-RGD coupling chemistry. RGD content ranged from 0, 30mg/g to 60mg/g. PEG content ranged from 0, 7mol% to 15mol%. Long term and short term cell culture was conducted to understand the effect of the PEG and peptide content for the optimization of the cell proliferation. There is no difference between mature HUVECs and ECFCs at 2 hours of incubation. However over longer time periods, the best performing polymers contained 7 mol% of PEG and 60mg/g RGD which supported sustainable growth of ECFCs without losing protein resistance. The thermoplastic polymers could be processed into fibrous scaffolds through electrospinning. Short and long term cell incubations were conducted. These materials retained their protein resistance and cell adhesion properties, indicating potential in fabricating scaffold or vascular grafts.

Since under physiological conditions, cells experience flow, the initial adhesion HUVECs and ECFCs was studied under shear flow conditions. The surface of a
variable shear-rate device was either coated with a polymer film or covered by synthetic fibers. Spin-coating was applied to produce smooth polymer films while fibrous scaffolds were generated by electro-spinning. A shear-rate-dependent increase of the attached cells with time was observed with both cell types. The adhesion of cells to surfaces is the result of competition between cell-surface interactions and hydrodynamic forces. RGD promotes cell adhesion and high shear rate reduces the number of attached cells. The number of attached ECFCs is significantly larger than that of HUVECs within the entire shear rate range and surfaces examined, especially on RGD linked polymers at low shear rates. Similar trends were observed on fibers. The superior adhesion characteristics of endothelial progenitor cells suggest they are a promising source for seeding vascular grafts under physiological conditions.

8.2 Future work

This dissertation describes the concept of a polymer system that can self endothelialize by harvesting EPCs from flowing blood. Previously adhesion of EPCs was achieved using novel peptides in the absence of serum. In this thesis PEG containing polymers with EPC specific peptides were synthesized which can overcome the problem of non specific protein binding that masks the active peptide sites. In future work, these strategies should be tested with whole blood under flow conditions. Although studies have been done with cell culture media, experiments with real blood and flow will allow determination of the surface characteristics necessary to produce a confluent EPC monolayer under physiological conditions.
Another challenge is to control the amount of peptide incorporated. From amino acid analysis, with fixed monomer ratios and temperature, the resulting peptide density in the polymers varied widely. The factors determining the peptide incorporation efficiency are not completely known. It is expected to that at high levels of peptide incorporation maximum enhanced cellular adhesion will occur. Further work should explore the variables that affect peptide incorporation rate such as the reaction time between succinimide and peptides. Furthermore, all peptide densities are reported as bulk concentrations. Since cell ligand interactions occur on the surface, advanced surface characterization techniques such as XPS and surface ion mass spectroscopy (SIMS) may be useful to measure the surface peptide densities.

In all experiments, only one type of cell was studied in cell culture media. Real blood is much more complex as it contains red and white blood cells, platelets and proteins. Phage display selected peptide performed well \textit{in vitro}. Whether or not our highest affinity ligands bound to non-fouling biomaterials can achieve EPC capture in actual blood circulation remains a big challenge for further investigation. Additionally evolving an \textit{in vitro} EPC monolayer so that endothelial cell phenotype is achieved should be a goal for future work. Accomplishing such a phenotypic change on EPCs harvested from whole blood would be the ultimate accomplishment.

There are also many other aspects requiring further study for the purpose of developing a vascular graft, such as mechanical properties, sutureability, etc. It is hoped that materials described in this research may be useful in developing devices that could passively accumulate \textit{in vivo} an autologous monolayer of highly
proliferative HBOECs that will ultimately lead to improve vascular prostheses or other devices that come into contact with blood.
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