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Development of Poly(Propylene Fumarate-co-Ethylene Glycol): An Injectable, Biodegradable Implant for Cardiovascular Applications

by

Laura J. Suggs

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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May, 1998
ABSTRACT

Development of Poly(Propylene Fumarate-co-Ethylene Glycol: An Injectable, Biodegradable Implant for Cardiovascular Applications

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Laura J. Suggs

A novel block copolymer consisting of poly(propylene fumarate), PPF, and poly(ethylene glycol), PEG, was fabricated and evaluated for use as a cardiovascular stent to prevent reclosure of the vessel lumen following balloon angioplasty. This copolymer has been fabricated in a block configuration with two to three homopolymer units in series through a transesterification reaction between the linear polyester and the terminal hydroxyl functionalities of the PEG. This material has been characterized in terms of structure and composition as well as thermal properties and solubility behavior.

We described the preparation and bulk characterization of crosslinked P(PF-co-EG) hydrogels. The extent of the crosslinking reaction and the degree of swelling in aqueous solution were determined on several different copolymer formulations. Mechanical properties were evaluated and were shown to increase with increasing PPF molecular weight and decrease with increasing PEG content. The degradation behavior was examined in vitro at pH 7.4 and in vivo in a subcutaneous rat model, in terms of mass loss, dimensional changes, mechanical properties, morphology, and biocompatibility over a twelve week time course. The P(PF-co-EG) hydrogels demonstrated a pattern typical of bulk degradation. They retained at least a 20% of their initial ultimate tensile stress after three weeks with no apparent changes in morphology.

Platelet adhesion and aggregation on P(PF-co-EG) hydrogels was examined under both static and flow conditions. We demonstrated a significant decrease in platelet attachment on the copolymer hydrogel films relative to PPF. In addition, there were also reductions in attachment resulting from an increase in PEG weight percent or molecular weight. The copolymer surfaces showed no thrombus formation or platelet spreading.
Cytotoxicity in culture and *in vivo* biocompatibility in a rat cage implantation model were also assessed. The copolymer hydrogels showed significant cytotoxicity *in vitro*, however, there was no evidence of inflammation *in vivo* due to toxic leachable products. The copolymer showed excellent biocompatibility with no evidence of foreign body giant cell formation. We have demonstrated the ability of this material to be injection polymerized, and preliminary studies show that cells can be incorporated into the copolymer matrix during crosslinking.
ACKNOWLEDGMENTS

First, and most importantly, I would like to thank my family for their love and support throughout my academic career. Without them I would never have had the courage to persevere.

To my labmates and friends, past and present, thank you for the help and encouragement. It has not always been easy, but I wouldn’t trade my experiences for anything in the world because of all of you.

Thank you to all the undergraduate research assistants who have worked with me. I have learned a lot about how to be an advisor and a friend, and I hope it was a positive experience for all of you.

I would also like to thank Dr. Anderson and the people in his lab. My time in Cleveland was an eye-opening experience, and I am still overwhelmed at the helpfulness and competency of everyone there.

A special thank you to the committee members, Dr. McIntire and Dr. West as well as the other collaborators who made this work possible including Dr. Yaszemski, Dr. Engel, and Dr. Alemany.

Last, but not least, I would like to thank Dr. Mikos. Your enthusiasm and encouragement challenged me to do my best, and I as I look over this work, I can’t help but think it was all worth it.
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LIST OF ABBREVIATIONS

1,6-HD Hexanediol
ANOVA Analysis of variance
AS-ODN Anti-sense oligodeoxynucleotide
ATR Attenuated total reflectance
BD 1,4-butanediol
BHPF Bis-hydroxypropyl fumarate
BP Benzoyl peroxide
BPB or MPM 1,2-propylene glycol dibutenoate
CABG Coronary artery bypass grafting
CHDI Cyclohexane diisocyanate
CPP Bis-(p-carboxyphenoxy)propane
ddH₂O Distilled deionized H₂O
DEPT Distortionless enhancement by polarization transfer
DMA Dynamic mechanical analysis
DMEM Dulbecco's modified eagle medium
DMT N,N-dimethyl-p-toluidine
DNA Deoxyribonucleic acid
DSC Differential scanning calorimetry
ED Ethylene diamine
EDTA Ethylenediaminetetraacetic acid
ESEM Environmental scanning electron microscopy
FBGC Foreign-body giant cell
FBS Fetal bovine serum
FTIR Fourier transform infrared
GPC Gel permeation chromatography
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-(N')-2-ethanesulfonic acid</td>
</tr>
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<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>IPN</td>
<td>Interpenetrating network</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>MDI</td>
<td>4,4'-diphenylmethane diisocyanate</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>P(D)LA</td>
<td>D enantiomer of poly(lactic acid)</td>
</tr>
<tr>
<td>P(D,L)LA</td>
<td>Enantiomeric mixture of D and L poly(lactic acid)</td>
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<td>p(HB-co-HV)</td>
<td>Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)</td>
</tr>
<tr>
<td>P(L)LA</td>
<td>L enantiomer of poly(lactic acid)</td>
</tr>
<tr>
<td>P(PF-co-EG)</td>
<td>Poly(propylene fumarate-co-ethylene glycol)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
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<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
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<td>PFP</td>
<td>Bis-propylene glycol fumarate</td>
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<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>PGPMMDMS</td>
<td>Poly(glycidoxy propyl methyl-dimethyl siloxane)</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly (3-hydroxybutyrate)</td>
</tr>
<tr>
<td>PI</td>
<td>Polydispersity index</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
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<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
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<td>PPF</td>
<td>Poly(propylene fumarate)</td>
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<td>PPP</td>
<td>Platelet-poor plasma</td>
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<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
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<tr>
<td>PSN</td>
<td>Penicillin/streptomycin/neomycin</td>
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<tr>
<td>PTCA</td>
<td>Percutaneous transluminal coronary angioplasty</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>PTMO</td>
<td>Poly(tetramethylene oxide)</td>
</tr>
<tr>
<td>SA</td>
<td>Sebamic acid</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>SR</td>
<td>Silicone rubber</td>
</tr>
<tr>
<td>t-CDM</td>
<td>Trans-cyclohexanediethanol</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TLC</td>
<td>Total leukocyte concentration</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethyl siloxane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VP</td>
<td>N-vinyl-2-pyrrolidinone</td>
</tr>
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<td>vWF</td>
<td>von Willebrand's factor</td>
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CHAPTER ONE

INTRODUCTION

1.1 Significance

Cardiovascular disease is a leading killer in the western world. Over 50% of deaths in the United States, Europe, and Japan can be attributed to cardiovascular disorders. The cost to the United States health care system to care for these disorders is estimated at over $9.3 billion. Atherosclerosis, a widespread type of cardiovascular disease, is often treated using balloon angioplasty. Angioplasties are somewhat successful; however, in 30-50% of angioplasties performed, the vessel reoccludes within 6 months, a process known as restenosis, and a repeat angioplasty or another procedure must be done to correct the problem. The cost of these repeat procedures to the United States health care system is estimated to be $5 billion dollars per year, in addition to an increased risk and inconvenience to patients.

1.2 Current Stenting Technologies

Coronary stents are currently used as a mechanical means to maintain patency in the diseased artery. These devices serve to guard against acute elastic recoil as well as to serve as a scaffold to contain torn or damaged arterial tissue in order to present a smooth surface to flowing blood. The majority of stents used clinically are metallic, and have been met with preliminary successes. There are, however, significant limitations to the current stenting technologies.
1.2.a Metallic Stents

The first implantation of a self-expanding, stainless steel stent in human coronary arteries was reported by Sigwart (Sigwart et al., 1987). Since then a number of different metals and designs have been employed to prevent restenosis. Stents can be broadly classified as one of three types. These include: balloon-expanding stents, self-expanding stents, and heat-expandable stents. Balloon-expanding stents require the insertion into the artery by a balloon catheter and subsequent inflation in order to achieve their full diameter. These include the stainless steel Gianturco-Roubin and Palmaz-Schatz stents, as well as the tantalum Strecker and Wiktor stents (Wright et al., 1985; Roubin et al., 1987; Palmaz et al., 1986; Schatz et al., 1987; White et al., 1988). Self-expanding stents are typically inserted by a guide catheter and require merely the removal of the catheter. The Wallstent and Z stent fall in this category (Sigwart et al., 1987). Heat-expandable stents require the use of a heat source in order to expand due to the nature of the titanium-nickel alloy known as Nitinol (Cragg et al., 1983).

Thrombogenicity remains one of the most significant problems with metallic stents. A number of factors can contribute to the thrombogenicity of biomaterial implants. These include: surface charge, surface free energy, critical surface tension, hydrophobicity, and surface configuration and roughness. Most metals have a known electropositive charge as well as a high critical surface tension. This contributes to the high thrombogenicity of most intravascular stents. In early studies, there was a high incidence of acute closure due to thrombosis. These problems were obviated through the use of a pharmacological regimen of anticoagulants and antithrombotic agents. Most metallic stents deployed clinically remain patent with the use of aspirin and warfarin therapies. It would be desirable, however, to employ a material that was inherently less thrombogenic.

Metallic stents are permanent implants, and as such evoke a foreign body reaction. This can result in the occurrence of intimal hyperplasia as described in Chp. 2. Neutrophils
and macrophages can infiltrate, secrete chemotactic factors, and then serve to recruit other proliferative cell types. The additional irritation of the metal against soft tissue may exacerbate the problem, and cause further inflammation, which results from a stent that is too stiff. Another related problem is compliance mismatch. This results from the mechanical differences between the stent and the surrounding tissue. The flow patterns are often disrupted in transition regions between the vessel and the implant. Areas of high shear stress are formed which have been shown to activate platelets and induce thrombosis and subsequent intimal hyperplasia.

Current modes of development include changes in geometry, filament thickness, roughness, and alloy composition. In addition, temporary metallic stents have been described (Schlarsky-Goldberg et al., 1990; Gaspard et al., 1991; Khorsandi et al., 1992). These types of stents may seem conceptually more appropriate to target a transient condition, however, the added trauma of removal may offset any advantages.

1.2.2 Polymer-Coated Metallic Stents

In 1988, Yoshioka reported a Gianturo-Roubin stainless steel stent which had been covered with an expandable mesh made of Nylon (Yoshioka et al., 1988). The polyamide acted to maintain the stent in position and avoid any distal migration. It also served to tact back the intimal flaps created by the ballooning process. In addition, it acted as a scaffold for neointimal growth to create a new vessel lumen. The combined thicknesses of this device, however, resulted in a small diameter for blood flow. Roeren described a Palmaz-Schatz stent coated with medical-grade silicone rubber (Roeren et al., 1990). Animal studies were performed in a rabbit model, and showed good initial patency. Long-term hyperplasia was still a problem. Polyurethanes have also been used to coat stainless steel stents (De Scheerder et al., 1994). Amphiphilic polyurethane-coated stents were studied in a porcine model and showed decreased acute thrombosis. In these attempts to render the
metallic surface more biocompatible, the problems with surface charge, critical surface tension, and roughness have been overcome to a certain extent, however, the permanent foreign body reaction and resulting hyperplasia have not.

In an effort to present a more natural surface to blood flow, fibrin, a natural polymer, was used to coat tantalum stents by Schwartz (Schwartz et al., 1992; Holmes, et al., 1994). A fibrinogen solution and a thrombin solution were dripped together onto a Wiktor stent. The resulting polymerization formed a layer of fibrin on the metal surface. The stents were evaluated in a porcine coronary model and showed reduced inflammatory response as evidenced by reduced multinuclear giant cell formation compared to polyurethane controls. This showed promise for reduction of arterial injury response and long-term restenosis. In addition, these coatings are resorbable and present the opportunity to incorporate drug delivery techniques in the form of polymeric microcapsules. Problems with natural polymer coatings include: donor source, immunological response, infection, and optimal composition.

Natural molecules and synthetic polymers have been used in conjunction, as in the case described by Sheth (Sheth et al., 1994). They reported coating Nitinol stents with a polyurethane-poly(ethylene oxide), PEO, copolymer onto which heparin had been covalently bound. The polyurethane served to bind the more hydrophilic polymer, PEO, to the stent. This group then acted as a mobile spacer to present heparin, a natural anticoagulant, to flowing blood. A rabbit carotid model was used to show better patency relative to uncoated stents. In a similar effort, Lincoff used a biodegradable polymeric coating, poly(L-lactic acid), in order to deliver dexamethasone to the coronary artery in a porcine model. Results showed no difference between coated and uncoated stents (Lincoff et al., 1994). Dexamethasone did not limit neointimal proliferation, however, this idea may be an effective method for the delivery of other more efficacious molecules.

One important limitation with drug-eluting polymer-coated stents appears to be the low volume which may be incorporated into the thin coatings. A high drug concentration
in the local area of injury may not be sustainable for extended periods. In addition, there are transport considerations with respect to the drug reaching tissues relative to flowing blood. Also, the drug must penetrate the tissue and retain its function. One study with a polyurethane-coated stent to deliver forskolin showed that tissue forskolin levels were proportional to the drug which was left in the stent coating (Eigler et al., 1994). The vasodilating and antiplatelet drug remained functionally active.

1.2.c Endothelialization and Gene Therapy

Studies have shown that as surrounding endothelium encases a metallic stent, thrombogenicity may be reduced (Schatz, 1989). In an effort to approximate this initially, Van der Giessen seeded human umbilical vein endothelial cells onto stainless steel stents in vitro (Van der Giessen et al., 1988). These experiments showed that a complete endothelial layer could be distributed on stents in culture. Current techniques for cell culture on metallic stents call for the deposition of fibronectin onto the surface prior to seeding. Another method of achieving endothelial cell coverage is, "sodding." This technique consists of cell isolation from adipose tissue capillaries resulting in very large cell numbers relative to those achieved from vein isolation. The stent can then be sodded at a confluent density. Problems with endothelial cell culture on cardiovascular implants have been reviewed (Callow, 1987). The most important of these include: complications over the donor source, identification of implanted cells versus autologous cells, and determination of the function and expression of implanted endothelial cells.

In an effort to ensure that implanted endothelial cells are performing an anticoagulant function, Dichek and others have used the techniques of gene therapy to alter endothelial cells prior to seeding onto stainless steel stents (Dichek et al., 1989). Retroviral-mediated gene transfer was used to induce expression of either β-galactosidase or human tissue-type plasminogen activator (t-PA). Results showed that the majority of the
cells remained on the stent after expansion in vitro. Expression of t-PA was shown to be high relative to β-galactosidase controls. In addition, the transfection of endothelial cells with β-galactosidase may allow for the identification of implanted cells over those which have migrated from the surrounding tissue by simple histochemical staining.

Others have also described the impregnation of biodegradable polymeric stents with recombinant adenovirus (Rajasubramanian et al., 1994). Compound stents of PLLA and PCL were fabricated using a casting/winding process. PEG was used to modify the polymer surface, and the stent was immersed in a solution of recombinant virus stock. The virus was an adenoviral vector coded to express β-galactosidase as a reporter gene and absorbed readily into the hydrated polymer. Successful gene transfer of the reporter gene was shown in a rabbit carotid artery model by post-mortem histochemical staining.

1.2.d Biostable Polymer Stents

Polymers have been used extensively in the cardiovascular system. Biostable polymers which have been used for various applications include: polycarbonates, polyethylene, polypropylene, poly(ethylene terephthalate) (PET), polytetrafluoroethylene (PTFE), polysiloxane, polyurethanes, and silicone rubbers. Advantages of polymers are that some of these materials have been shown to have good biocompatibility relative to metals. This is due to the ability to fabricate smooth, noncharged polymer surfaces. In addition, their surface characteristics and mechanical properties can be specifically engineered to fulfill certain design requirements. Controlled drug delivery systems can also be incorporated into polymeric stents to achieve sustained localized dosages. Important considerations are the material composition itself which affects the mechanical properties as well as inherent thrombogenicity and the ability to reendothelialize. Polymeric stents could be engineered to be flexible enough to avoid problems with stiffness and compliance
mismatch while maintaining an intact lumen. Stent design is also an important consideration with respect to platelet activation and adhesion, and polymers can be easily processed in a number of configurations.

Polymer stents have previously been used for implantation in biliary and urinary tracts to treat obstructions. Materials used include: PET, PTFE, and polyethylene. PTFE stents have also been used for lymphovenous anastomosis (Shaper et al., 1992). Stents in these physiological areas do not have the stringent biocompatibility and antiplatelet requirements as those for implantation in the cardiovascular system. The primary function filled by these materials is a mechanical one.

Murphy designed a flexible, self-expanding PET stent and implanted it in porcine coronary arteries (Murphy et al., 1990; Murphy et al., 1992). This mesh stent was inserted in a collapsed state inside a delivery sheath. The removal of the sheath caused the deployment of the expanded stent. The PET material showed appropriate mechanical properties, however, the implant led to inflammation and proliferation which resulted in the reocclusion of the arteries. These tests did show a feasible mechanism for polymer stent implantation, and illustrate the importance of material selection. Van der Geissen developed a different configuration of PET stent based on a braided mesh (Van der Giessen et al., 1992; Van der Giessen et al., 1993). An in vivo study in a porcine model showed five of six correctly implanted stents remained patent.

2.1.e Biodegradable Polymer Stents

Biodegradable stents have certain advantages over those fabricated with other non-degradable materials. They have the potential to remain in the vessel transiently keeping the vessel patent, and then degrade into non-toxic products which can be excreted or resorbed by the body. The ability to sustain the vessel for a predetermined period of time and negate the potential need for a surgical removal procedure may be very advantageous. Important
aspects of design include the timing of degradation as well as the mode of degradation. Mechanical properties typically change with degradation, and this phenomenon can be controlled by material composition. Also, the release profiles of drugs can be controlled through compositional modifications.

Agrawal proposed a cardiovascular stent composed of poly(L-lactic acid) (Agrawal et al., 1992). It was fabricated in the form of a braided mesh, and the mechanical properties as a function of design parameters was assessed. The annealing temperature, the stent diameter, and the filament draw ratio were all varied in an effort to optimize the mechanical properties. It was shown that the maximum hydrostatic pressure decreased with increasing diameter and draw ratio. One important feature of polymeric materials versus metals is that polymers typically exhibit viscoelastic behavior. This means that there is not a linear relationship between stress and stent diameter. Optimal designs, however, maintained a collapse pressure above 300 mmHg. This stent design was tested in a canine femoral model. Results showed that the stents remained patent and did not cause significant inflammation or thrombosis. Degradation mode and changes in mechanical properties during degradation, however, appear to remain problematic.

Heat-expandable stents have also been proposed using polycaprolactone (PCL) (Slepkian and Schindler, 1988). PCL has a glass transition temperature at 52°C. This means that the polymer can be shaped to fit the vessel wall at temperatures above the glass transition temperature and then cooled to form a mechanically stable stent. The heating and shaping can be performed in situ by a number of different means. The first is through the use of a hot balloon technique in which a balloon is filled with a heated saline solution. Secondly, the balloon can be electrically heated, and third, heat can be generated through the use of microwaves. Additionally, these types of stents can be used in areas of complex geometry, not only in the vasculature, but also in bile ducts or bronchi due to the nature of the implantation. One drawback to the current technology is the heat release during the
shaping process. Temperatures up to 70°C can be reached, and this increase can damage tissue and cause necrosis at the site of implantation.

Other types of degradable polymers have been proposed for use as intravascular stents. These include: poly(glycolic acid) and its copolymers with PLLA, polyorthoesters, polyanhydrides, and poly(hydroxy butyrate/valerate). These polymers are discussed in detail in Chp. 2. Recently, a multilayered stent was proposed to address a number of design requirements (Eury, 1986). This stent was made up of a number of the above mentioned polymers each layer of which fulfilled a specific function. Certain layers were present to impart structural integrity while others controlled drug release. The configuration and nature of drug in each of the polymer layers was important in controlling drug release. The outer layers would degrade quickly and target anti-thrombogenic therapies, while the inner layers would provide a more controlled release for the prevention of intimal hyperplasia. Many of these polymers have also been tested for their response in a porcine coronary model (Lincoff et al., 1992). Results from this study conclude that degradable polymer coatings on stainless steel stents elicited a greater proliferative response compared to uncoated controls. Clearly, more work needs to be done in examining polymers and polymer compositions.
CHAPTER TWO

BACKGROUND

2.1 Cardiovascular Biology

2.1.a Tunica Intima

Blood vessels all have many structural similarities in common as shown in Figure 2.1. They are composed of three tunics, or layers. The first layer is the tunica intima which is a one-cell thick layer directly in contact with blood flow. This layer is composed entirely of endothelial cells, which are of mesenchymal origin. They are polygonal and typically measure 10x30 μm on their flat surface. Being in contact with flowing blood causes them to elongate in the direction of flow. Endothelial cells taper towards the connection with neighboring cells. These connections are perforated with zonula occludens junctions and have variable permeability. These junctions are of physiological importance in allowing passage of fluid, in the case of edema, and extravasation of cells, in the case of inflammation. The endothelial cells rest on a layer of loose connective tissue. This tissue can also be populated with a few migratory smooth muscle cells. This layer serves to maintain a non-thrombogenic surface in the vasculature.
Figure 2.1. Tunics (or layers) of which the arterial wall is composed.
2.1.b Tunica Media

The second layer common to all blood vessels is the tunica media. The media is a composite layer consisting of smooth muscle cells in a fibrous matrix. There are up to 40 cell layers in larger arteries. The matrix is composed of elastin, collagen, and proteoglycans. The smooth muscle cells themselves produce the matrix in which they are embedded, and again, the fibers are oriented in the direction of flow. One difference in vessel structure is in the case of arteries. The intimal and medial layers in arteries are separated by a thin layer of elastin fiber known as the internal elastic lamina. The gaps or fenestrae which are found in the lamina allow for the passage of nutrients to the vessel tissue. Elastin and collagen fibers which compose the media serve to impart strength as well as compliance to the vessel wall both of which are important in the contractile artery where pressure is higher. Proteoglycans serve as signals to migratory cells and can also serve as soluble signals when liberated from the matrix through digestion.

2.1.c Tunica Adventitia

The adventitia is composed of elastin and collagen fibers along with connective tissue. The connective tissue is primarily fibroblasts and a few adipocytes. In the arteries, there is an elastic layer thinner than the internal elastic lamina called the external elastic lamina. The collagen is different between the respective layers with primarily type III in the media and type I in the adventitia. The vasa vasorum, or vessels which nourish the artery, are found throughout the adventitia. These vessels provide nutrients to the tissue of the media and adventitia, as these layers are too thick to be nourished by diffusion from the main vessel alone. Blood vessels are also enervated with unmyelinated sympathetic nerve fibers. The nerve fibers and vasa vasorum are often grouped together in the adventitial layer.
2.2 Atherosclerosis

Atherosclerosis is a ubiquitous pathology occurring in children as young as 10 years old (Stary, 1989). The process of atherosclerosis is thought to begin with some type of injury to the endothelial cells that line the blood vessel (Ross, 1993). This injury may be mechanical, such as shearing of the cells; or chemical, including exposure to molecules such as oxidized low-density lipoprotein (LDL). In response to this injury, endothelial cells initiate the healing process. As part of this healing, endothelial cells secrete agents that increase the adherence of macrophages and T lymphocytes. These cells then migrate between the endothelial cells and locate in the intima. The macrophages accumulate lipids and become large foam cells. These cells then accumulate with T cells and smooth muscle cells to form a fatty streak which will eventually become the fibrous atherosclerotic plaque (Figure 2.2). As the lesions grow by accumulating more cells, some of the foam cells may migrate back into the lumen. At this point, these sites can become thrombogenic. The ultimate result is the recruitment of several cell types such as endothelial cells, smooth muscle cells, platelets, and macrophages which all release cytokines and growth factors that interact to produce uncontrolled growth of cells, primarily smooth muscle cells. This phenomenon is known as intimal hyperplasia.
Figure 2.2. Schematic of a fibrous atherosclerotic plaque.
Current therapies to treat atherosclerosis generally work to prevent thrombosis at the site of vessel narrowing to prevent occlusion of the vessel. Platelet inhibitors and anticoagulants serve this function. In order to prevent the initial plaque forming event, lipid-lowering drugs are also used in some cases. However, there are some patients who do not respond to treatment or first come in for treatment in the advanced stages of disease. In these cases, interventional procedures must be performed. There are several interventional procedures which mechanically manipulate the atherosclerotic lesion to restore flow to the vessel. The most common procedure is percutaneous transluminal coronary angioplasty (PTCA) in which a catheter with a deflated balloon is inserted into the artery. Once at the site of the lesion, the balloon is inflated to stretch the vessel. Other techniques include atherectomy and laser or rotary ablation in which the plaque is cut out of the artery. The most invasive and serious procedure is coronary artery bypass grafting (CABG). This is done in the most serious cases in which several arteries are affected or initial procedures have failed.

2.3 Restenosis

Restenosis, like atherosclerosis, may be described as aggressive wound healing (Forrester et al., 1991). During the angioplasty procedure, the artery is injured by a number of means. First, in up to 30% of angioplasties, part or all of the endothelial layer is denuded or removed, due to friction from the catheter itself. Also, in many procedures, the atherosclerotic plaque cracks and cuts the artery, breaches the internal elastic lamina, and sometimes inflicts injury as far as the medial layer. In either case, the endothelial monolayer is disrupted, which can lead either to acute reocclusion or long-term restenosis.
2.3.a Acute Closure

Following balloon angioplasty, there may be a spontaneous narrowing of the vessel lumen, known as elastic recoil. A diseased vessel can often contain a plaque which is located predominately to one side. The inflation of the balloon typically stretches the non-calcified, elastic region of tissue. After dilation, the compliant segment recoils to its original diameter, thus negating any benefit from the transient dilation. In addition, the vessel has been damaged, which compounds the impedance in flow caused by the tissue recoil.

The disruption of the endothelial layer initiates a cascade of pathological events. The first being the exposure of subendothelial components to flowing blood. These components can include lipids, collagen, elastin, fibronecctin, and vitronectin. In normal vasculature, the endothelium releases compounds which act to maintain the non-thrombogenic surface and relax the vessel. These compounds include heparan sulfate, thrombomodulin, tissue plasminogen activator, prostacyclin, and nitric oxide. The cracks or flaps formed from injury may serve as impedances to blood flow, and both chemical and mechanical influences cause platelet activation and subsequent adherence to the denuded vessel wall. Platelet accumulation and aggregation is primarily mediated by the glycoprotein IIb/IIIa receptor. Thrombin is then secreted by activated platelets and initiates pathways which convert fibrinogen to fibrin, thus resulting in the formation of a thrombus. Thrombus formation along with intimal or medial flaps and/or elastic recoil can cause reocclusion of the vessel immediately following PTCA.

2.3.b Chronic Restenosis

If the thrombus formation immediately following balloon inflation does not cause acute closure, additional events can result in procedural failure. Platelets contain storage
granules know as Weibel-Palade bodies which store factors important in the clotting process, one of which is von Willebrand's factor (vWF) or factor VIII. In addition, platelets also release factors which have been implicated as mitogens and chemotactic factors. Platelet-derived growth factor (PDGF), for example stimulates the migration of leukocytes as well as promoting the dedifferentiation of smooth muscle cells (SMCs) to a secretory phenotype. Platelets have also been shown to release thromboxane, serotonin, thrombospondin, and transforming growth factor β (TGFβ). These substances all serve to stimulate growth of SMCs. Leukocytes which have been recruited, including neutrophils and macrophages as a result both of platelet recruitment and injured SMCs, also secrete a number of growth factors. Macrophages, for example, secrete PDGF, basic fibroblast growth factor, TGF β, interleukin-1, and heparin-binding epidermal growth factor. This hyperplastic growth continues for approximately 1 to 6 months.

The initial injury itself has also been shown to stimulate the transformation of myocytes in the media to a more fibroblastic phenotype, possibly due to the disruption of the matrix surrounding the muscle cells. The transformed SMCs along with fibroblasts some from as far away as the adventitial layer begin to migrate towards the intima. They then begin to proliferate extensively and secrete additional extracellular matrix proteins. Proteoglycans are produced including: chondroitin sulfate, dermatan sulfate, and heparan sulfate. After 2 to 3 months, the SMCs appear to return to a more quiescent, contractile state, and the proteoglycans which make up the matrix are gradually replaced by collagen, elastin, and fibronectin. Meanwhile, endothelial cells have grown from the edges of the damaged tissue to cover the hyperplastic formation. A diagram of the progression of restenosis is given in Figure 2.3.
Figure 2.3. Diagram showing the progression of restenosis.
The result of intimal hyperplasia and arterial wall remodeling is again the renarrowing or restenosis of the vessel lumen. This phenomena in itself can cause significant circulatory difficulties. This, along with the formation of thrombus can completely reocclude the artery up to six months after an interventional procedure.

2.3.c Restenosis Therapies

Because of the complex nature of restenosis, no therapy for restenosis has been as successful as hoped. Current procedures include prolonged inflation at the time of angioplasty, placement of a stent into the artery as described previously, and treatment with antiplatelet drugs (Zidar et al., 1994). Each of these therapies has advantages and disadvantages. The prolonged inflation means that the procedure time must be lengthened which places the patient at higher risk. Stents provide mechanical support to prevent closure of the vessel, but there are problems with thrombosis on the surface. Drug therapy has seemed promising in animal trials, but due to problems with animal models, no drug therapy has proven successful in humans. Types of pharmacological agents include: antiplatelet agents, antithrombotic agents, calcium-channel blockers, angiotensin-converting enzyme inhibitors, antimitogenic agents, hypolipidemic agents, and growth factor inhibitors.

Research is active in this area, and gene therapy is one emerging area that is being investigated. Both endothelial cells and SMCs are potential targets for gene therapy (Nabel et al., 1991). Genes could potentially be delivered in vivo, or cells could be removed from the patient, transfected with the appropriate gene, and then reinfused into the patient at the time of angioplasty. Endothelial cells would most likely be used in this case to restore the non-thrombogenic layer that is injured during angioplasty. Also, these same cells are also being used to coat stents, as previously described. Gene transfer into endothelial cells and
SMCs has been demonstrated in animal models with retroviral vectors, adenoviral vectors and liposome-mediated gene delivery.

There are several potential sites where gene therapy could be used. Some researchers are investigating adding genes to endothelial cells or SMCs that will code for anti-platelet agents to prevent the initial aggregation of platelets. Others are studying ways to inhibit SMC proliferation by blocking genes, with anti-sense oligodeoxynucleotides (AS-ODNs), that are required for cell growth. These latest studies are in early stages, so there are no human clinical trials at this time.

2.4 Biodegradable Polymers

2.4.a Poly(a-Hydroxy Esters)

2.4.a.1 Poly(Glycolic Acid)

Poly(glycolic acid) (PGA) is a highly crystalline, hydrophilic, linear aliphatic polyester (Structure 1). As such, it has a high melting point and a relatively low solubility in most common organic solvents. At room temperature, PGA is soluble in hexafluoroisopropanol, a highly toxic solvent.
Structure 1:
Poly(glycolic acid)

\[
\begin{array}{c}
\text{O} \\
\text{CH}_2 - \text{C} \\
\end{array}
\]

Structure 2:
Poly(lactic acid)

\[
\begin{array}{c}
\text{O} \\
\text{CH} - \text{C} \\
\text{CH}_3 \\
\end{array}
\]

Structure 3:
Poly(e-caprolactone)

\[
\begin{array}{c}
\text{O} \\
\text{(CH}_2)_5 - \text{C} \\
\end{array}
\]

Structure 4:
Poly(ortho ester)

\[
\begin{array}{c}
\text{O} \\
\text{O} - \text{R} \\
\end{array}
\]

Structure 5:
Poly(ortho ester)

\[
\begin{array}{c}
\text{R}' \\
\text{O} - \text{CH}_2 \\
\text{C} \\
\text{O} - \text{CH} - \text{R} \\
\end{array}
\]

Structure 6:
Poly(ortho ester)

\[
\begin{array}{c}
\text{R}' \text{CH}_2 \\
\text{OCH}_2 \\
\text{C} \\
\text{CH}_2 \text{O} \\
\text{O} - \text{R} \\
\end{array}
\]

Structure 7:
Polyanhydride

\[
\begin{array}{c}
\text{O} \\
\text{C} - \text{R} - \text{O} \\
\end{array}
\]
Structure 8:
Polyanhydride

Structure 9:
Poly(3-hydroxybutyrate)

Structure 10:
Poly(3-hydroxyvalerate)

Figure 2.4. Structures of several polymers currently being investigated for use as components of cardiovascular stents.
PGA degrades primarily by bulk erosion. This occurs through random hydrolysis of its ester bonds. Reed and Gilding report that this degradation has a bimodal distribution with the first phase of degradation occurring by diffusion of water to the amorphous regions and subsequent hydrolysis (Reed and Gilding, 1981). The second phase begins as water penetrates and hydrolyzes the more crystalline regions. The degree of crystallinity, therefore, affects the degradation rate. The molecular weight distributions which show two degradation phases are given in Figure 2.5 (Reed and Gilding, 1981). Mass loss occurs primarily during the second phase completing the entire process between weeks 4 and 12 for PGA surgical sutures. The rate of hydrolysis in vitro can be controlled by varying the pH (Chu, 1981). A more basic or acidic environment drives hydrolytic cleavage. In addition, the degradation rate can be affected by the polymerization time or "curing time" of PGA, as shown in in vivo studies (Miller et al., 1977).

The crystallinity of PGA is typically between 46% and 52% (Reed and Gilding, 1981). The maximum crystallinity during degradation occurs in the region between the two degradation phases. These values are influenced by the cooling or "quenching" process as well as the molecular weight of the polymer (Cohn et al., 1987).

PGA (crystallinity 50%) loses most of its mechanical strength over the first 2-4 weeks of degradation (Reed and Gilding, 1981). This is asynchronous with the mass loss which begins at approximately week 4. This is due to the bimodal degradation distribution. The amorphous regions are hydrolyzed first which results in loss of mechanical strength, while the degradation and diffusion of low molecular weight chains later result in significant mass loss. The stress/strain curves showing the effect of degradation on mechanical strength are given in Figure 2.6 (Reed and Gilding, 1981).
Figure 2.5. Molecular weight distributions as a function of time for PGA sutures at pH 7 and 37°C showing the bimodal degradation distribution.
Figure 2.6. Stress/strain curves showing the loss of mechanical strength with degradation for PGA sutures at pH 7 and 37°C.
2.4.a.2 Poly(Lactic Acid)

Poly(lactic acid) (PLA) (Structure 2) is also a linear polyester, but the presence of an extra methyl group makes it more hydrophobic than PGA. Its water uptake in thin films is approximately 2% (Gilding and Reed, 1979). The methyl group contributes to a more amorphous character as well as increasing its solubility in organic solvents. In addition, this group creates a chiral center which contributes to three different forms of the polymer. These are abbreviated as follows: P(D)LA, P(L)LA, and P(D,L)LA. The mostly commonly used form is PLLA which, when degraded, releases the biological product, lactic acid. PLA is frequently cast from common solvents. These include: chloroform, methylene chloride, methanol, ethanol, benzene, acetone, dioxane, dimethylformamide, and tetrahydrofuran (Younes and Cohn, 1987; Gogolewski and Pennings, 1982; Gogolewski and Pennings, 1983).

PLA has also been shown to degrade by homogeneous, hydrolytic erosion (Chawla and Chang, 1985; Pistner et al., 1993; Kulkarni et al., 1971), however, there is some evidence that enzymatic reactions are involved (Spenlehauer et al., 1989) which is due primarily to the difference in crystallinity, and enzymes may also have an effect on PGA in the latter stages of degradation. PDLA degrades in a more conventional two stage process where the majority of molecular weight loss occurs in the first stage, and the subsequent loss in mass and tensile strength begins in the second stage at a number average molecular weight of 15,000 (Reed and Gilding, 1981). PLLA of molecular weight 95,000 degraded in vivo by 56% in 6 months based on peak molecular weight (Mp) (Chu, 1982). For PDLA of Mp between 58,000 and 87,000, 49% degraded in vivo in 1 month. A half-life of 6.6 months was reported (Miller et al., 1977) for PLLA of molecular weight 85,000. In vitro studies (Chu, 1982) showed a 50% loss in weight average molecular weight (Mw) in 16 weeks with a concurrent loss of 10-15% by mass. The degradation rate of PLA also varies with varying pH (Vert et al., 1991; Von Recum et al., 1995).
Thermal and mechanical properties of both PLLA and PDLA of various molecular weights are given in Tables 2.1 and 2.2, (Holland and Tighe, 1986) as well as additional properties (Jamshidi et al., 1988).

2.4.a.3 Poly(Lactic-co-Glycolic) Copolymers

The advantage of copolymerizing poly(a-hydroxy esters) is the ability to control physical and mechanical properties, however, there is no linear relationship between the physical properties of the constituent homopolymers and their copolymers. Most of these copolymers are amorphous (between approximately 24 and 67 mol % PGA) (Gilding and Reed, 1979), and therefore, degradation rates are highly dependent on the relative amount of each comonomer. Copolymers with high or low copolymer ratios are much more stable to hydrolytic attack than copolymers with a more equimolar ratio. Half-lives for various ratios are depicted graphically in Figure 2.7 (Miller et al., 1977).

Due to the dependence on pH of poly(a-hydroxy esters), a phenomenon known as autocatalysis occurs where the carboxylic acid monomers released during degradation reduce the pH and further induce degradation (Vert et al., 1991; Von Recum et al., 1995; Vert et al., 1994). For large-scale polymers, autocatalysis causes a heterogeneous degradation where the pH decreases in the center of the polymer, and a differential in the degradation rate is created (Vert et al., 1994).

Multiple uses of poly(lactic acid), poly(glycolic acid), and their copolymers have been described (Frazza and Schmitt, 1971; Agrawal et al., 1992; Lewis, 1990; Mikos et al., 1994).
<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_w$</th>
<th>$T_g$ (°C)</th>
<th>$T_m$ (°C)</th>
<th>$T_d$ (°C)</th>
<th>$H_f$ (J g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ortho ester)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-CDM:1,6-HD=35:65</td>
<td>99,700</td>
<td>55</td>
<td>-</td>
<td>358</td>
<td>Amorphous</td>
</tr>
<tr>
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<td>Amorphous</td>
</tr>
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<td>-</td>
<td>254</td>
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</tr>
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<td>-</td>
<td>255</td>
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<td></td>
</tr>
<tr>
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<tr>
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<tr>
<td>11 mol% HV</td>
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<td>12</td>
</tr>
<tr>
<td>22 mol% HV</td>
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<tr>
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$M_w$ = Weight average molecular weight  
$T_g$ = Glass transition temperature  
$T_m$ = Melting temperature  
$T_d$ = Decomposition temperature  
$H_f$ = Enthalpy of fusion

Table 2.1. Thermal properties of synthetic biodegradable polymers.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>$M_w$</th>
<th>Tensile Strength (MPa)</th>
<th>Tensile Modulus (MPa)</th>
<th>Flexural Modulus (MPa)</th>
<th>Elongation at Yield (%)</th>
<th>Elongation at Break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(ortho esters)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>t-CDM:1,6-HD=35:65</td>
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<td>820</td>
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<tr>
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<td>800</td>
<td>1000</td>
<td>4.1</td>
<td>180</td>
</tr>
<tr>
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<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Poly(lactic acids)</td>
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<td></td>
</tr>
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<td>2350</td>
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<tr>
<td>Poly(hydroxybutyrate)</td>
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<tr>
<td>0 mol% HV</td>
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<tr>
<td>7 mol% HV</td>
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<td>1400</td>
<td>1600</td>
<td>2.3</td>
<td>2.8</td>
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<tr>
<td>11 mol% HV</td>
<td>529,000</td>
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<td>620</td>
<td>750</td>
<td>8.5</td>
<td>36</td>
</tr>
<tr>
<td>Poly(e-caprolactone)</td>
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<td>16</td>
<td>400</td>
<td>500</td>
<td>7.0</td>
<td>80</td>
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</tbody>
</table>

$M_w$ = Weight average molecular weight

Table 2.2. Mechanical properties of several biodegradable polymers.
Figure 2.7. Variation of half-life with copolymer ratio of PLGA in vivo.
2.4. Poly(e-Caprolactone)

Poly(e-caprolactone) (PCL) is a semi-crystalline, aliphatic polyester (Structure 3). It is soluble in tetrahydrofuran, chloroform, methylene chloride, carbon tetrachloride, benzene, toluene, cyclohexanone dihydropyran, and 2-nitropropane; and only partially soluble in acetone, 2-butane, ethyl acetate, acetonitrile, and dimethyl fumarate (Pitt, 1990). PCL is also capable of forming blends as well as useful copolymers with a wide range of polymers (Koleske, 1978).

PCL has been shown to degrade by random hydrolytic scission of its ester groups, and under certain circumstances, by enzymatic degradation (Pitt, 1990). It is similar to PDLA, in that it degrades in a two-phase process with the molecular weight loss occurring primarily in the first phase, and the major mass and strength loss at the onset of the second at a number average molecular weight of 5,000 (Pitt et al., 1981). However, PCL degrades almost three times slower than PDLA (Engelberg and Kohn, 1991). A graph of molecular weight versus time showing the degradation of PCL capsules in vivo is given in Figure 2.8 (Pitt et al., 1981). The crystallinity of PCL increases with decreasing molecular weight with polymers of molecular weight above 100,000 being about 40% crystalline. This value increases to about 80% for molecular weights of 5,000 (Pitt et al., 1981). As a result, PCL behaves like PGA in that the residual crystallinity increases as the polymer degrades. In addition, PCL is affected by acidic conditions consistent with an autocatalytic degradation mechanism, and it is also influenced by the addition of small molecules such as ethanol, pentanol, oleic acid, decylamine, and tributylamine (Pitt and Gu, 1987).

PCL has a low $T_g$ of -60°C and a melting temperature range of 59-64°C. It is always in a rubbery state at room temperature. It has been postulated that this leads to a high permeability of PCL for controlled release agents. Other thermal and mechanical properties are listed in Tables 2.1 and 2.2.
Figure 2.8. Degradation of PCL capsules loaded with various drugs *in vivo*.
2.4.c Poly(Ortho Esters)

Poly(ortho esters) are amorphous, hydrophobic polymers containing hydrolytically labile, acid-sensitive, backbone linkages (Structures 4, 5, and 6). Due to their hydrophobicity, they can easily dissolve in organic solvents including chloroform, methylene chloride, and dioxane. However, it can be difficult to remove the solvent in a situation such as a solvent casting (Heller et al., 1990). In addition, these polymers are not inherently susceptible to the presence of water, although they can be if anhydrides (acid excipients) have been incorporated. They are susceptible to thermal degradation and must be processed accordingly. Sparer (Sparer et al., 1984) reported significant degradation at 160°C for a linear polymer of 3,9-bis(ethylidene-2,4,8,10-tetraoxaspiro(5,5)undecane) and a 75:25 mole ratio of 1,6-hexanediol/trans-cyclohexanedimethanol (1,6-HD/t-CDM) compounded with an anhydride.

Poly(ortho esters) are a class of polymers which can degrade heterogeneously by surface erosion (Heller, 1993). These polymers lose material from the surface only, while retaining their original geometric shape. As such, their primary use is in drug delivery (Sparer et al., 1984). The first poly(ortho ester), as shown in Structure 4, generates a carboxylic acid upon hydrolysis which then further catalyzes the acid-sensitive cleavage. A basic salt such as Na2CO3 or Mg(OH)2 is usually incorporated to neutralize the acid product, however, this creates a diffusion-limited system which exhibits non-zero-order drug release characteristics.

The second class, represented by Structures 5 and 6, does not produce acidic hydrolysis products, and its degradation can be controlled by adding either acidic or basic excipients. In the case of acid addition, water penetrates, ionizes the acid, and reduces the pH. This then catalyzes the hydrolysis, resulting in a hydration front and an erosion front. For a basic excipient, water must penetrate, elute or neutralize the base, and then allow erosion to occur, decreasing the rate of hydrolysis (Heller, 1990). According to the choice
of additive, degradation rates can be varied from several days to years. Acid excipients can also be incorporated into the polymer itself as pendant chains which are solubilized upon cleavage (Shih et al., 1984). The polymer can also be cross-linked at temperatures as low as 40°C with an excipient-stabilized interior (Heller, 1985). A graph of the degradation rates of various poly(ortho esters) is given in Figure 2.9 (Heller et al., 1985).

The mechanical and thermal properties of these polymers can also be varied over a wide range by the selection of starting materials with differing compositions and molecular weights. A graph of the glass transition temperature versus percent diol is given in Figure 2.10 (Heller et al., 1983). Other thermal and mechanical properties for a selected group of poly(ortho esters) are listed in Tables 2.1 and 2.2.

2.4.d Polyanhydrides

Polyanhydrides are a class of hydrolytically unstable polymers that are usually either aliphatic, aromatic, or a combination of the two (Domb et al., 1993). Two general representations are given in Structures 7 and 8. These polymers dissolve in common organic solvents including chloroform and methylene chloride and are extremely sensitive to aqueous environments. In addition, they are very reactive and can react with amine or other nucleophilic groups that are present in drugs intended for controlled release. This is true especially in polymer processing at elevated temperatures (Gopferich, 1996).
Figure 2.9. Cumulative weight loss with time for poly(ortho esters) (in the form of 6x0.5 mm disks at pH 7 and 37°C). ▼ = 58% t-CDM, 38% 1,6-HD, 4% DHSA; ○ = 59% t-CDM, 39% 1,6-HD, 2% DHSA; □ = 59.5% t-CDM, 39.5% 1,6-HD, 1% DHSA; △ = 59.7% t-CDM, 39.75% 1,6-HD, 0.5% DHSA.
Figure 2.10. Glass transition temperatures for various percentages of 1,6-hexanediol of 3,9-bis(ethylidene-2,4,8,10-tetraoxaspiro(5,5)undecane) with trans-cyclohexane dimethanol and 1,6-hexanediol.
The degradation of polyanhydrides can be varied from days to years depending on
the choice or combination of choices of backbone structure. The degradation rate of several
different combinations of the aliphatic monomer, sebacic acid (SA), and the aromatic
monomer, bis-(p-carboxyphenoxy)propane (CPP), is given in Figure 2.11 (Chasin et al.,
1990). The polymer primarily degrades by surface erosion (Gopferich and Langer, 1993;
Rosen et al., 1983; Tamada and Langer, 1993). As such, it has been targeted as a
candidate for drug delivery, eliminating the need for additional excipients. Its degradation
rate is also sensitive to changes in pH, typically increasing with increasing pH as shown in
Figure 2.12 (Chasin et al., 1990).

There is a wide variety of processing techniques available for forming
polyanhydrides, however, care must be taken in incorporating controlled release agents at
high temperatures because of the reactivity of the polymer with the drug and the instability
of the polymer itself. The mechanical properties of polyanhydrides are generally poor,
tending to be brittle with minimal fiber-forming abilities. Attempts at increasing these
properties include crosslinking with a copolymer of fumaric acid as well as forming
copolymers which contain imide bonds. In addition, polyanhydrides have been shown to
have excellent in vivo biocompatibility (Laurencin et al., 1990). A detailed presentation of
thermal properties is given (Tamada and Langer, 1992).

2.4.e Poly(3-Hydroxybutyrate) and Copolymers

Poly (3-hydroxybutyrate) (PHB) is a crystalline, thermoplastic polyester made by
microorganisms as an energy storage molecule (Structure 9). As such, it can be
enzymatically degraded by certain bacteria. It is often copolymerized with hydroxyvaleric
acid (Structure 10) to create poly(3-hydroxybutyrate-co-3-hydroxyvalerate), p(HB-co-
HV). Solvent casting has been described from solution in chloroform, methylene chloride,
and tetrahydrofuran (Wang et al., 1990; Holland et al., 1987).
Figure 2.11. Effect of time on cumulative percent of polyanhydride degradation for different CPP and SA copolymers in the form of compression-molded disks of 1.4 cm diameter and 1 mm thickness at pH 7.4 and 37°C.
Figure 2.12. Effect of time on cumulative percent of polyanhydride degradation at varying pH levels for CPP and SA copolymers in 1.4 cm dia./1 mm thick compression-molded disks at pH 7.4 and 37°C.
The degradation of PHB produces D-3-hydroxybutyric acid, normally found in human blood, which may contribute to its low toxicity. There is evidence for both enzymatic and hydrolytic degradation in vivo (Gogolewski et al., 1993). In vitro studies (Wang et al., 1990; Holland et al., 1987) suggest that PHB and p(HB-co-HV) copolymers degrade by hydrolysis in a multi-stage process where the majority of the molecular weight loss occurs before any significant mass loss. A graph of weight loss for various p(HB-co-HV) copolymers is given in Figure 2.13 (Holland et al., 1987). The copolymerization of hydroxybutyric acid with hydroxyvaleric acid increases the percentage of amorphous regions compared to PHB which are readily attacked by hydrolytic degradation thereby increasing degradation rates. In addition, elevated temperatures and alkaline conditions have been shown to increase degradation rates.

The crystallinity and mechanical properties of the p(HB-co-HV) copolymer can be varied by modification of the percentages of the respective monomers. The higher the percentage of hydroxyvalerate, the less crystalline and the more elastic the polymer becomes. Some thermal and mechanical properties are given in Tables 2.1 and 2.2. A study of thermal characteristics in vivo is given, and a mechanical evaluation in vivo and in vitro is also given (Gogolewski et al., 1993; Miller and Williams, 1987).
Figure 2.13. Percent of initial weight loss with time for solvent-cast disks of p(HB-co-HV) copolymers with 2 cm diameter and 0.15 mm thickness at 70°C and pH 7.4. + = 10% HV, $M_w = 750,000$; × = 20% HV, $M_w = 300,000$; Δ = 12% HV, $M_w = 170,000$; □ = 12% HV, $M_w = 100,000$; Δ = 20% HV, $M_w = 36,000$. 
2.5 Poly(Propylene Fumarate)

Poly(propylene fumarate) (PPF) is a degradable polymer which is being developed by a number of groups for use as an orthopaedic implant. It is a linear polyester which degrades into fumaric acid, a component of the Kreb's cycle, and propylene glycol, which has been used as a plasma expander. PPF has unsaturated sites along the polymer backbone which allow for addition crosslinking through the use of a vinyl monomer. Such monomers include: N-vinyl-2-pyrrolidinone (VP), methyl methacrylate, hydroxyethyl methacrylate, and others. It is being developed as an alternative to the current bone cement, poly(methyl methacrylate) (PMMA), which is not degradable and remains in the body as a permanent implant. In addition, the crosslinking of PMMA occurs at a very high temperature. This curing causes damage to the surrounding tissue and subsequent necrosis around the implant. A degradable alternative which could fill a non-uniform defect, provide structural support during the healing process, induce bone ingrowth, and degrade upon completion of its function would be desirable.

Several synthesis methods have been proposed for the fabrication of PPF and other related polyesters. In 1976, Wise reported the fabrication of polymers consisting of acids comprising the Kreb's cycle with biocompatible polyols (Wise, 1976). Acids included citric, cis-aconitic, isocitric, α-ketoglutaric, succinic, fumaric, amalic, and oxaloacetic. The invention exclusively used glycerol as the second component, however, mannitol and sorbitol were listed as possible alternatives. The reactions were performed by solution polymerization in dimethylformamide and suspension polymerization with fumaric or other acids and α-monoacetin at temperatures ranging from 145-153°C with various catalysts. Synthesis was also performed using succinic anhydride and glycerol with para-toluenesulfonic acid as a catalyst at 163°C.
Sanderson reported the synthesis of PPF in 1988 by an esterification. (Sanderson, 1988). This method used diethyl fumarate and propylene glycol (PG) as the starting reagents. They were heated at 240-250°C with para-toluenesulfonic acid as a catalyst. The reaction caused the nucleophilic by the attack of the carbonyl group on the fumarate ester and displaced ethanol which evolved at a temperature of 78°C. The resulting polymer was characterized by viscometry and gel permeation chromatography in THF. Ninety percent of the polymer had molecular weights within 1,300-124,000. The PPF made by this method was crosslinked using benzoyl peroxide as a radical initiator and VP as the vinyl monomer. A calcium sulfate filler was also used in the crosslinking formulation to create a moldable paste which could be formed into irregularly-shaped defects prior to curing.

Gerhart reported a composite for use as a biodegradable bone cement (Gerhart and Hayes, 1989). They fabricated PPF though a condensation reaction with propylene glycol and fumaric acid. The reaction was heated to 145°C for five hours and then increased to 180°C. The product had weight-average molecular weights between 1500 and 4200 and number-average molecular weights between 500 and 1200. This material was crosslinked using methyl methacrylate as a crosslinking monomer, benzoyl peroxide as an initiator with dimethyl-p-toluidine (DMT) as an accelerator. Composites were formed using a solid phase consisting of calcium phosphate ceramics and bioresorbable calcium salts. These serve to impart initial mechanical strength, induce bone ingrowth, and then elute from the matrix to leave space for cellular proliferation. Compressive strengths of 19 MPa were achieved. In vitro degradation studies showed minimal decrease in mechanical properties after 3 weeks (Gerhart et al., 1988).

Domb proposed several synthesis methods based on the formation of several multimeric starting reagents. These include: two intermediate trimers, bis-propylene glycol fumarate (PFP) and 1,2 propylene glycol dibutenoate (BPP or MPM), and propylene glycol oligomers (Domb, 1989). PFP was produced through a reaction with fumaric acid and propylene oxide in solution for 20 hrs using pyridine as a catalyst followed by
neutralization and washing. MPM was produced in a solution precipitation reaction with maleic anhydride and propylene glycol at 100°C. The PPF polymers were then synthesized in three main ways. The first was the synthesis of PPF using non-volatile glycols which included the following schemes: the reaction of PFP or MPM trimers or pentamers with fumaric acid, the reaction of PFP with diethyl fumarate, the direct reaction of PFP and MPM trimers and pentamers, the reaction of PFP, MPM, or fumaric acid with propylene glycol oligomers. The second way was through step polymerization of PFP or MPM trimers with maleic anhydride or propylene oxide, and the third way was the direct reaction of propylene glycol and fumaric acid similar to that described by Gerhart.

Domb also studied the effects of both the PPF molecular weights and the PPF terminal groups on the mechanical properties of the resulting crosslinked composites. The highest weight-average molecular weight achieved was 1890 with a polydispersity of 1.4. The purity reported, however, was only 65% (Domb et al., 1990). Composites were formed using methyl methacrylate, and benzoyl peroxide as an initiator. A calcium filler consisting of tricalcium phosphate and calcium carbonate was added along with DMT as an accelerator. Compression testing was performed and the highest strength, at 6.9 MPa, was obtained by the high molecular weight PPF. PPF with terminal vinyl and epoxide end groups were assessed in terms of mechanical properties and degradation behavior (Domb et al., 1996). Mechanical strengths between 30 and 129 MPa were achieved, and after 4 weeks in buffer solution, the composites retained compressive strengths above 20 MPa.

Gresser synthesized PPF using fumaric acid and propylene glycol with p-toluenesulfonic acid monohydrate as a catalyst as described by Sanderson. (Gresser et al., 1995). An inhibitor, t-butyl hydroquinone was added to prevent premature crosslinking. Number-average molecular weights of 2600 were achieved with polydispersities of 2.6. Composites were formed from PPF, VP, tricalcium phosphate, and DMT. They examined how compositional variations affect the extent of crosslinking, and they achieved over 90% PPF incorporation.
Yaszemski produced PPF through a two-step reaction as shown in Figure 2.14, first forming an intermediate, and then employing a transesterification reaction to create the polymer (Yaszemski et al., 1995; Yaszemski et al., 1996). In the first step, fumaryl chloride was reacted with propylene glycol to produce bis(2-hydroxypropyl fumarate) at room temperature in the absence of any catalyst. The intermediate was purified through solution-precipitation, and was then reacted at 160°C under vacuum in a nitrogen environment. Propylene glycol was removed by distillation. The resulting polymer was again purified through solution-precipitation and dried. Number average molecular weights of 1225 with polydispersities of 1.6 were achieved without the use of a catalyst during the transesterification step after 30 hrs of reaction time. Using antimony trioxide as a catalyst, number average molecular weights of 1500 with polydispersities of 3.

Peter identified two additional saturated products from this reaction scheme (Peter et al., 1997c). One resulted from HCl addition across the double bond in the fumarate group. The second resulted from acid-catalyzed addition of an ROH group across the double bond. The backbone structure was investigated through the use of Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopies. Kinetic studies were performed and a number average molecular weight of 1500 with a polydispersity of 2.8 was achieved. *In vitro* and *in vivo* degradation studies were performed using the composite formulation described by Yaszemski. Compositional differences were also examine for their effects on degradation and mechanical properties. Compressive strengths *in vitro* exhibited an initial maximum value of 2.6 MPa, reaching a value of 3.24 MPa after seven weeks in buffer solution. *In vivo* results showed a maximum compressive strength of 7.7 MPa initially, with no mechanical integrity by 6 weeks (Peter et al., 1997b; Peter et al., in press).
Figure 2.14. Synthesis of PPF as described by Yaszemski et al.
Peter synthesized higher molecular weight PPF, through the reaction of fumaryl chloride and propylene glycol in the presence of potassium carbonate, which served as a proton scavenger as shown in Figure 2.15 (Peter et al., submitted). Transesterification of the resulting low molecular weight oligomer led to a polymer with greater molecular weights than previous reaction methods without requiring the use of a catalyst. According to two dimensional NMR, the backbone structure of this polymer was as expected and contained no byproducts formed by acid-catalyzed addition across the fumarate double bond. Kinetic studies of the transesterification showed that the molecular weight reached a final number average molecular weight of 4900 after 16 hrs, while the polydispersity index remained below 1.8 throughout the reaction. The crosslinking characteristics of a composite formed from this material were also investigated (Peter et al., submitted). It was shown that the maximum crosslinking temperatures ranged from 38-48°C.

2.6 Poly(Ethylene Glycol) Copolymers

Poly(ethylene glycol) is a unique molecule which exhibits unusual solubility behavior. It is soluble in organic solvents as well as being very hydrophilic. It is also terminated with easily-derivatized hydroxyl groups, and these qualities make it an attractive molecule for many different reaction schemes. Also, it has been shown that PEG of low molecular weights can be excreted by the kidneys without significant accumulation in the liver or spleen (Herold et al., 1989). In addition, the extreme hydrophilicity of PEG makes it nonthrombogenic and nonadhesive to cells, and as such, it has been covalently bound to a number of different polymers in order to reduce cellular and platelet adhesion for use as biomedical implants.
Figure 2.15. Synthesis of PPF as described by Peter et al.
A number of different copolymers with PEG have been proposed for medical applications. Merrill described the development of segmented polyurethanes with PEG as the soft segment and the hard segment from cyclohexane diisocyanate (CHDI) and ethylene diamine (ED) (Merrill et al., 1982). PEG of molecular weights 600-8000 was reacted with CDHI in solution with dibutyl tin dilaurate as a catalyst at 60°C. ED was then added to extend the chain. Platelet retention studies were performed and showed reduced platelet retention relative to the polyurethane control. Silver also looked at the haemocompatibility of polyurethanes containing PEG (Silver et al., 1994). They investigated a PEG polymer consisting of 4,4'-diphenylmethane diisocyanate (MDI) and 1,4-butanediol (BD) as the hard segment in an ex vivo canine model. Similar PEG molecular weights were used, and it was determined that only the polymers made with PEG of molecular weight 600 were significantly more thrombogenic.

Polyurethaneureas with polyol soft segments have also been fabricated by Grasel based on MDI and ED (Grasel and Cooper, 1986). These materials were evaluated as blood-contacting materials in an ex vivo canine model. Results showed compatibility variations with changes in hard/soft segment ratios. The more phase separation, the lower the platelet deposition. Takahara also examined a similar segmented polyurethaneurea based on MDI, ED, and poly(tetramethylene oxide) (Takahara et al., 1991). They used a two-step polymerization method, where polyethylene oxide-polypropylene oxide copolymer diols were reacted with MDI at 75°C with a stannous octoate catalyst. PTMO was then added for 1 hour, and the solution cooled to room temperature. ED was added and the temperature was again raised to 75°C. This technique formed a segmented polyurethaneurea with hydrophilic side chains. Ex vivo studies showed that a more thrombogenic response correlated with an increase in the concentration of hydrophobic side chain.

Chaikof examined platelet interaction in interpenetrating networks (IPNs) of polyethylene glycol and poly(glycidoxy propyl methyl-dimethyl siloxane) (PGPMDMS)
(Chaikof and Merrill, 1990). This copolymer was fabricated through the endlinking reaction of the hydroxyl group of the PEG with the epoxy group on the derivatized polysiloxane. IPNs were then formed from the cationic polymerization of the epoxy end groups using a boron trifluoride etherate producing simultaneous endlinking of PEG and polymerization of polysiloxane sequences. *Ex vivo* shunt experiments in a baboon femoral model showed reduced platelet deposition for molecular weights above 8,000 and for those polymers fabricated with PEG contents of 65%.

Materials based on polyether/polyester copolymers have also been studied for medical applications. Gilding synthesized poly(ethylene glycol-co-ethylene terephthalate) through a transesterification form di(2-hydroxyethyl) terephthalate monomer and PEG using antimony trioxide and a catalyst (Gilding and Reed, 1979). They evaluated PEG molecular weights of 600 and 1540 at weight percentages of 50, 60, and 70. Mody synthesized a copolymer of PEG with poly(butylene terephthalate) (Mody and Wilkes, 1981). The synthesis was performed by a condensation reaction between dimethyl terephthalate, butanediol, and 5,5-dimethyl-1,3-poly(oxyethylene)hydantoin. Other PEG copolymers include those with polyimides and polystyrene which have also been synthesized in an effort to increase the *in vivo* compatibility of these materials (Pathak et al., 1994; Grainger et al., 1989).

Hydrolytically degradable copolymers based on poly(α-hydroxy esters) with PEG have also been proposed. Zhu described a triblock copolymer of poly(ε-caprolactone) (PCL) and PEG (Zhu et al., 1986). This was performed through the anionic polymerization of PCL and alkali metal aldoxide derivatives of PEG. Other have made copolymers of PLA and PEG through the polyesterification reaction of lactic acid in the presence of PEG chains using an antimony trioxide catalyst (Cohn and Younes, 1988). Still others have described copolymers of PLGA and PEG terminated with acrylate groups (Sawhney et al., 1993). The PEG hydroxyl termini were used as ring-opening groups to
copolymers could be UV polymerized in contact with tissues, and results showed minimal cellular adhesion *in vitro*.

These PLGA/PEG acrylate copolymers have been investigated as blood/tissue barriers to reduce restenosis (Hill-West et al., 1994; West and Hubbell, 1996). This technique is known as paving and requires the surface polymerization of thin hydrogels in order to mask the damaged lumen from flowing blood. The crosslinked hydrogel is highly hydrophilic and as such is very nonthrombogenic. This technique was studied in a rat arterial crush model and a rat balloon injury model. It was also evaluated in a rabbit balloon model in which it inhibited thrombosis and reduced intimal thickening by approximately 80%. The degradation times of the materials were less than 24 hrs, however, it was determined that early blood-borne signals were important factors in initiating intimal hyperplasia.
CHAPTER THREE

OBJECTIVES

The goal of this project was to develop a material for use as a cardiovascular stent for the prevention of restenosis following balloon angioplasty. The intention was to design a polymer which fulfilled certain mechanical and biological functions while facilitating the delivery of endothelial cells in order to repave the damaged arterial lumen. Several design requirements needed to be addressed to achieve this goal. The stent itself must be easily implanted with minimal damage to the surrounding tissue. The stenting material must maintain a certain level of mechanical integrity over a predetermined period of time in vivo in order to prevent elastic recoil. The material must have good biocompatibility. It must be non-thrombogenic to avoid platelet adhesion and aggregation for the prevention of thrombosis. In addition, it must also show a minimal inflammatory response in contact with tissues in order to avoid complications with intimal hyperplasia. Finally, since no biomaterial is completely inert, we would like this material to be able to deliver bioactive molecules or cells to the damaged area in order to encourage reendothelialization.

We hypothesized that a block copolymer consisting of poly(propylene fumarate) and poly(ethylene glycol) could be developed which has the advantageous properties of both the parent homopolymers. The PPF component, through the fumarate double bond, would confer the ability to be crosslinked in situ at a relatively low temperature. This would allow the material to be injection polymerized into the arterial space with minimal damage to the lumen of the artery as well as the distal path through which the catheter was inserted. The ester linkage of the PPF blocks would also allow the resulting material to be susceptible to ester hydrolysis resulting in bioreabsorbable degradation products. The PEG component is extremely hydrophilic, and its incorporation into the copolymer backbone would result, upon crosslinking, in a hydrogel. PEG hydrogels are known to be non-
thrombogenic and biocompatible and can facilitate diffusion of bioactive molecules. Through alterations in the copolymer composition and molecular weight, we further hypothesized that the mechanical and biological properties of the material could be tailored for its particular application.

We fabricated the P(PF-co-EG) copolymer and verified the structure and connectivity of the two homopolymers. A number of design parameters were varied in order to assess their effect on the properties of the resulting copolymer. This material characterization was performed not only to determine structure and composition but also to quantify thermal properties and solubility behavior of the linear molecule. We further described the preparation and bulk characterization of the crosslinked P(PF-co-EG) hydrogel. It was important to examine the extent of the crosslinking reaction and the degree of swelling in aqueous solution for a swellable hydrogel intended for in vivo use. Determination of mechanical properties both in tension and dynamic flexion was performed. We wanted to establish that the physical properties of P(PF-co-EG) hydrogels could be tailored for specific applications by altering the material composition. The mode of degradation of these hydrogels and how well the mechanical properties were maintained were important considerations. Their mass loss, dimensional changes, mechanical properties, morphology, and biocompatibility over a twelve week time course were evaluated.

Platelet adhesion and aggregation on P(PF-co-EG) hydrogels was described under both static and flow conditions in order to evaluate the thrombogenicity of the copolymer hydrogels. In addition, changes in coverage resulting from the increase in poly(ethylene glycol), PEG, weight percent or molecular weight were tested to see how the material could be optimized. The cellular response in vitro and in vivo to the presence of copolymer hydrogels was evaluated. One important factor in the implantation of biomaterials is toxic leachable products. We assessed the cytotoxicity of copolymer hydrogels to cultured endothelial cells. For the in vivo study, we used a cage implant system to evaluate the
inflammatory response of unleached copolymer films. This gave us information about how the material interacted with leukocytes and to the extent of inflammation over a three week time course. Finally, we wanted to evaluate the ability of P(PF-co-EG) hydrogels to serve as an injectable carrier for endothelial cells. Important considerations are the cytotoxicity of the material as described above as well as the temperature of crosslinking. \textit{In vivo} studies were also performed to verify the ability of the copolymer to crosslink \textit{in situ}. 
CHAPTER FOUR

SYNTHESIS AND CHARACTERIZATION OF A BLOCK COPOLYMER
CONSISTING OF POLY(PROPYLENE FUMARATE) AND POLY(ETHYLENE
GLYCOL)

4.1 Introduction

In this chapter we describe the synthesis and characterization of P(PF-co-EG) in terms of molecular, thermal, and solubility properties, all of which are important considerations in the development of an in situ polymerizable system (Suggs et al., 1996; Suggs et al., 1997a; Suggs et al., 1997b). In order to assess the effects of different design parameters upon physico-chemical properties, a fractional factorial design has been employed (Box et al., 1978). This statistical design allows the evaluation of the degree to which a design parameter affects resulting properties while performing a minimal number of experiments. This approach allows not only the characterization of the copolymer but also provides information about how changing reaction parameters can affect the material properties.

4.2 Experimental Section

4.2.a Polymer Synthesis

Prior to synthesis, the starting reagents, fumaryl chloride (Aldrich, Milwaukee, WI) and propylene glycol (Acros, Pittsburgh, PA) were distilled with boiling ranges of 161-164°C and 186-188°C, respectively. All other chemicals and reagents were used as received from the manufacturer.
PPF was produced as previously described (Peter et al., 1997c). Briefly, fumaryl chloride was added dropwise to a three fold molar excess of propylene glycol at room temperature in the absence of any catalyst. HCl was given off as a gas and trapped in aqueous NaOH. The resulting intermediate fumaric diester was then purified by solution-precipitation in chloroform and petroleum ether, respectively. The second step was a transesterification of the intermediate to form the linear polyester. Finally, the copolymer was formed by adding poly(ethylene glycol) (Aldrich, Milwaukee, WI) to the reaction vessel at 160°C under a vacuum of approximately 115 mm Hg. Antimony trioxide was added as a basic catalyst and propylene glycol was removed by condensation. The entire reaction was performed under nitrogen by the use of a sparging apparatus within the reaction vessel. The extent of the copolymerization reaction was monitored using thin layer chromatography as described below. The resulting copolymer was again purified by solution-precipitation as described above. Excess chloroform and petroleum ether were removed by roto-evaporation. Formation of the unsaturated copolymer is shown in Figure 4.1.

![Reaction scheme for the formation of P(PF-co-EG).](image-url)

Figure 4.1. Reaction scheme for the formation of P(PF-co-EG).
4.2.b Thin Layer Chromatography

Thin layer chromatography was used to monitor the disappearance of the PEG homopolymer. Samples of the copolymer were taken every 2 hours during the reaction. These samples were then dissolved at a concentration of about 2% (w/v) in chloroform and filtered. Silica gel plates were spotted with 1 mL of this solution and were eluted in 25% (v/v) pyridine in tetrahydrofuran. The plates were then visualized with a 1% solution of iodine in methanol.

4.2.c Gel Permeation Chromatography

The homopolymer and copolymer molecular weight distributions as well as the copolymer compositions were determined by GPC using a differential refractometer (Waters, Model 410, Milford, MA) and a tunable absorbance detector or ultraviolet detector (Waters, Model 486) connected in series. A Phenogel guard column (50x7.8 mm, 5mm, mixed bed, Phenomenex, Torrance, CA) and a Phenogel column (300x7.8 mm, 5mm, mixed bed, Phenomenex) were used to elute the samples at 1 mL/min chloroform flow rate. Polystyrene standards were used to obtain a universal calibration curve, and this was then used to calculate the polymer molecular weights.

In addition, the concentrations and compositions of the copolymers were determined by evaluating the total area output of both detectors (Balke, 1991). The area output from each of the starting homopolymer standards was assumed to be directly proportional to the concentration in order to create a number of calibration curves. The concentration of each type of homopolymer in the block copolymer was then determined from the peak area of the copolymer signal. The composition of each copolymer could be calculated as well as the amount of PPF which had been incorporated. The average number of PEG blocks in each copolymer formulation, \( \bar{b} \), was also determined based on the
copolymers number average molecular weight, $\bar{M}_{n,\text{copolymer}}$, the starting PEG number average molecular weight, $\bar{M}_{n,\text{PEG}}$, and the weight fraction of PEG, $w$. Number average molecular weight was used as described by Yan and Yuan (Yan and Yuan, 1987).

$$b = \frac{\bar{M}_{n,\text{copolymer}}}{\bar{M}_{n,\text{PEG}}} w$$  \hspace{1cm} (4.1)

4.2.d Fourier Transform Infrared Spectroscopy

Infrared spectra were taken on a Nicolet 550 spectrometer (Madison, WI). All copolymers were analyzed using a zinc selenide ATR crystal. The resolution of the instrument was specified as 4 cm$^{-1}$ at a wavenumber of 1000 cm$^{-1}$.

4.2.e Nuclear Magnetic Resonance Spectroscopy

Proton and carbon NMR spectra were acquired on a Bruker AC250 NMR spectrometer ($^1\text{H} = 250$ MHz, $^{13}\text{C} = 62.9$ MHz) using CDCl$_3$ solutions containing TMS. To obtain $^1\text{H}$ spectra with accurate relative signal intensities, conservative conditions were used: 9.3-µs 90° pulse, 2994 Hz (12.0 ppm) spectral width, 10.945 s FID acquisition time, and 5 s relaxation delay before repeating the sequence to improve signal-to-noise. No line broadening was used to process the FID. DEPT-135 $^{13}\text{C}$ parameters for the PPF and copolymer samples were: 10,638 Hz (169 ppm) spectral width, 3.08 s FID acquisition time with low power proton decoupling (WALTZ-16 sequence with 109 µs 90° $^1\text{H}$ pulse), and 4 s relaxation delay before repeating the sequence. Line broadening of 0.3 Hz was used to process the FID and improve signal-to-noise. The DEPT-135 $^{13}\text{C}$ parameters for the PEG samples were: a 5,495 Hz (87 ppm) spectral width and a 2.98 s FID acquisition time.
4.2.f  Differential Scanning Calorimetry

A TA Instruments model 2920 Modulated DSC with a Mechanical Cooling Accessory (Newcastle, DE) was used to measure melting points, glass transition temperatures, and heats of fusion for all of the polymer compositions. The sample cell was calibrated with indium ($T_m$=429.8K) and filled with dry helium in order to optimize heat transfer.

The glass transition temperatures were determined from thermal scanning after quenching to -70°C from a 70°C melt and then ramping up to 70°C again at 15°C/min. Melting temperatures were found after annealing from 70°C to -50°C at 1°C/min. This was done in order to bring the sample below the crystallization temperature, and then scanning was performed at 10°C/min to 100°C. All samples weighed between 4 and 6 mg. Both the onset and midpoint of the $T_g$ and the onset and maximum of the $T_m$ were recorded.

The heat of fusion, $\Delta H_m$, in cal/g was also recorded, and the degree of PEG crystallization, $X$, was determined from the following equation:

$$X = \left(\frac{\Delta H_m}{\Delta H_m^c w}\right) \times 100$$

(4.2)

where $w$ is the weight fraction of PEG in the sample (as determined by GPC) and $\Delta H_m^c$ is the theoretical heat of fusion of 100% crystalline PEG (49 cal/g) (Chaikoff and Merrill, 1990).

4.2.g  Solubility Determination

Polymers were placed in 9 different solvents including: water, chloroform, tetrahydrofuran, acetone, dimethyl formamide, methanol, toluene, cyclohexane, and ethyl
ether. Approximately 0.03 g of sample was dissolved in 3 mL of solvent to create a 1% solution and allowed to sit for 24 hours at room temperature.

4.2.h Statistical Design

Several parameters were varied in order to assess their effects on the properties of the copolymer. This was accomplished through the use of a resolution IV, two level, fractional factorial design (Box et al., 1978). The parameters varied were as follows: PEG nominal molecular weight was 2,000 or 4,600, transesterification time for the PPF homopolymer was 5 hours or 10 hours, copolymer transesterification time was 5 hours or 10 hours, and weight percent PEG was 33% or 66%. The design is given in Table 4.1. In total, eight copolymers were made.

4.3 Results and Discussion

4.3.a Molecular Characterization

The copolymerization reaction times were chosen such that the PEG could react completely with the PPF, which was in excess in terms of reactive groups. The extent of reaction was monitored by TLC. All of the PEG was shown to disappear within 4 hours, and although this technique is primarily qualitative, we and others (Horii et al., 1975) have found TLC to be sensitive down to a 1% homopolymer concentration in a copolymer mixture.
<table>
<thead>
<tr>
<th>Run Number</th>
<th>Nominal Molecular Weight of PEG</th>
<th>PPF Reaction Time</th>
<th>Copolymer Reaction Time</th>
<th>% PEG by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,000</td>
<td>5 hours</td>
<td>5 hours</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>2,000</td>
<td>5 hours</td>
<td>10 hours</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>2,000</td>
<td>10 hours</td>
<td>5 hours</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>2,000</td>
<td>10 hours</td>
<td>10 hours</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>4,600</td>
<td>5 hours</td>
<td>5 hours</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>4,600</td>
<td>5 hours</td>
<td>10 hours</td>
<td>66</td>
</tr>
<tr>
<td>7</td>
<td>4,600</td>
<td>10 hours</td>
<td>5 hours</td>
<td>66</td>
</tr>
<tr>
<td>8</td>
<td>4,600</td>
<td>10 hours</td>
<td>10 hours</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 4.1: Resolution IV fractional factorial design showing the eight combinations of high and low values for four different design parameters.
The molecular weights of the starting materials as well as the molecular weights and compositions of the resulting copolymer as determined by GPC are given in Table 4.2. The molecular weights of the starting PEG polymers are significantly higher than those of the nominal values. This is due to the fact that polystyrene standards were used, and calibration curves were generated using Mark-Houwink constants of $a=1$ and $K=0$, in order to remain consistent and since the constants for the copolymer were not known. A sample chromatogram is given in Figure 4.2. The high molecular weight polymer peak which occurred from 5.8 to 7.6 minutes is the copolymer peak from which the composition was determined. The low molecular weight peaks from 7.6 to 11.6 minutes represent the PPF which had not been converted, based on chromatograms of the PPF homopolymer (not shown). The molecular weights of the resulting polymers as compared to the parent homopolymers show that, at most, they are diblock or triblock copolymers, which could explain the bimodal copolymer peak. Additionally, the average number of PEG blocks in each copolymer, $\bar{b}$, support this conclusion in that there are between one and two PEG blocks in each copolymer formulation.

The effects of the design parameters described in Table 4.1 on the percentage of PPF which is incorporated into the copolymers are shown in Figure 4.3. Increasing the reaction time of the starting PPF homopolymer from 5 hours to 10 hours increases the percentage of PPF incorporated by increasing its relative length in the copolymer chain. Increasing the weight percent of PEG from 33% to 66% increases the percentage of PPF incorporated due to the increase in hydroxyl groups that are available to react, while increasing the weight average molecular weight of PEG from 4570 to 10,700 decreases the percentage of PPF incorporated due to the relative decrease in available hydroxyl groups. Increasing the copolymerization reaction time from 5 hours to 10 hours has no significant effect due to the fact that all the PEG has already reacted.
Figure 4.2. Sample GPC chromatogram showing the copolymer peak at an elution time of 6-7 minutes along with the unconverted PPF homopolymer.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>$\overline{M}_w$ of Copolymer/PEG$^1$</th>
<th>$\overline{M}_n$ of Copolymer/PEG$^1$</th>
<th>$\overline{M}_w$ of pPF$^2$</th>
<th>$\overline{M}_n$ of pPF$^2$</th>
<th>% PEG in Copolymer</th>
<th>% PPF Converted</th>
<th>PEG Block Number, $\overline{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 2K</td>
<td>4570 ± 40</td>
<td>4050 ± 30</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PEG 4.6K</td>
<td>10700 ± 90</td>
<td>9170 ± 370</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PPF 5 hours</td>
<td>—</td>
<td>—</td>
<td>1200 ± 20</td>
<td>700 ± 20</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PPF 10 hours</td>
<td>—</td>
<td>—</td>
<td>4120 ± 20</td>
<td>1780 ± 10</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Copolymer 1</td>
<td>11170 ± 120</td>
<td>8200 ± 150</td>
<td>900 ± 70</td>
<td>530 ± 20</td>
<td>71</td>
<td>70</td>
<td>1.4</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>9400 ± 30</td>
<td>7370 ± 10</td>
<td>1460 ± 120</td>
<td>860 ± 40</td>
<td>58</td>
<td>51</td>
<td>1.1</td>
</tr>
<tr>
<td>Copolymer 3</td>
<td>10660 ± 20</td>
<td>8050 ± 20</td>
<td>1540 ± 40</td>
<td>1010 ± 90</td>
<td>47</td>
<td>55</td>
<td>0.9</td>
</tr>
<tr>
<td>Copolymer 4</td>
<td>14060 ± 40</td>
<td>10060 ± 90</td>
<td>1070 ± 130</td>
<td>690 ± 150</td>
<td>65</td>
<td>83</td>
<td>1.6</td>
</tr>
<tr>
<td>Copolymer 5</td>
<td>14500 ± 30</td>
<td>13090 ± 20</td>
<td>1770 ± 30</td>
<td>930 ± 50</td>
<td>74</td>
<td>21</td>
<td>1.1</td>
</tr>
<tr>
<td>Copolymer 6</td>
<td>16340 ± 40</td>
<td>13950 ± 40</td>
<td>1340 ± 40</td>
<td>750 ± 30</td>
<td>81</td>
<td>49</td>
<td>1.2</td>
</tr>
<tr>
<td>Copolymer 7</td>
<td>16740 ± 40</td>
<td>14200 ± 70</td>
<td>1810 ± 20</td>
<td>1050 ± 40</td>
<td>77</td>
<td>59</td>
<td>1.2</td>
</tr>
<tr>
<td>Copolymer 8</td>
<td>15890 ± 100</td>
<td>14081 ± 160</td>
<td>2310 ± 40</td>
<td>1330 ± 30</td>
<td>59</td>
<td>30</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^1$ Determined from either the copolymer peak or the starting PEG homopolymer.

$^2$ Determined from either the unconverted PPF peak or the starting PPF homopolymer.

Table 4.2. Molecular weights and compositions of P(PF-co-EG) copolymers.
Figure 4.3. Magnitude of the change on the percent PPF that is incorporated into the copolymers caused by the increasing the values of the four different design parameters. (n=3, standard error=4.6%)
The FTIR spectra of copolymers 1 and 5 as well as representative spectra of the starting homopolymers are given in Figure 4.4. The carbonyl stretch occurs at 1725 cm\(^{-1}\) for PPF and copolymers 1 through 4. For copolymers 5 through 8 this peak shifted slightly to 1721 cm\(^{-1}\). This peak moved to lower wavenumbers with increasing molecular weight of the starting PEG. This is probably due to vibrational coupling which is known to depend upon the length of the polymer chain (Koenig, 1992; Silverstein et al., 1991). The bands at 959 and 842 cm\(^{-1}\) are known to be characteristic of the crystalline phase of PEG (Bailey and Koleske, 1976). The peak at 959 cm\(^{-1}\) is absent in copolymers 2 and 3, and the intensity relative to the crystalline bands is lowered in copolymers 5 and 8. Both of these findings correlate well with the DSC results. The peak at 759 cm\(^{-1}\) was assigned to the amorphous phase of the copolymer.

The \(^1\)H NMR spectrum of copolymer 4 is given in Figure 4.5. The peaks of the expected functional groups can be assigned as follows based on 2-D and DEPT-135 spectra (Koenig, 1992; Silverstein et al., 1991) of the intermediate fumaric diester (not shown): olefinic protons at 6.8-7.0 ppm, methyl protons at 1.0-1.6 ppm, and two sets of terminal methine and methylene protons from the addition of the propylene glycol in two possible orientations. The secondary alcohol gives propyl methine protons and propyl methylene protons both at 4.1 ppm. The primary alcohol gives propyl methine protons at 5.2 ppm and propyl methylene protons at 3.8 ppm (Peter et al. 1997c). The internal propyl group on the polymer gives methylene protons at 4.1-4.3 ppm and methine protons at 5.2-5.4 ppm. The main ethylene peak is found at a chemical shift of 3.65 ppm with peaks of the ethylene protons near the ester linkage at 3.7 and 5.4 ppm. Products have also been determined which result in the partial saturation of this molecule and which complicate the spectra (Peter et al., 1997c). The formation of these products prohibits quantitative analysis of the copolymer composition based on NMR.
Figure 4.4. FTIR spectra of copolymer formulations 1 and 5 as well as representative homopolymer spectra showing the frequencies of several important peaks. The spectra were acquired neat and are plotted on a common scale and are offset by 0.5 absorbance units.
Figure 4.5. $^1$H NMR spectrum of copolymer 4 in CDCl$_3$. 
The DEPT-135 $^{13}$C spectra of the starting PEG homopolymer with a weight average molecular weight, $M_w$, of 4,570, copolymer 4, and PEG-diacylate (Aldrich, Milwaukee, WI) are given in Figures 4.6, 4.7, and 4.8, respectively. The spectra of the parent PEG homopolymer shows the interior methylene group carbons all resonating at 70.4 ppm. The three carbon atoms adjacent to the terminal hydroxyl groups resonate at 70.1, 72.6, and 61.2 ppm. In the $^{13}$C spectrum of copolymer 4, the internal methylene carbons are present at 70.5 ppm, while those peaks representing the terminal carbons are absent or their intensity is greatly reduced relative to the internal peaks. In addition, two new peaks are formed that are not present in either of the starting materials. In order to determine whether these peaks were the result of the terminal ester linkage on the PEG, the spectra of a model compound of PEG-diacylate was acquired. The internal methylene carbons are present at 70.5 ppm with some residual hydroxy-terminated signals at 70.2, 72.6, and 61.5 ppm. The methylene groups near the ester linkage resonate at 63.6 and 69.0 ppm which correspond well to the new peaks in the copolymer at 64.4 and 68.8 ppm. The peak at 66.5 ppm appears to be an impurity.
Figure 4.6. DEPT-135 $^{13}$C NMR spectra of PEG with $\overline{M}_w=4,570$ in CDCl$_3$. 
4.3.b Thermal Analysis

The melting temperatures, glass transition temperatures, heats of fusion, and percentages of crystalline PEG as determined by DSC are given in Table 4.3. Each of the copolymers exhibited a melting peak, although the peaks for the copolymer formulations in which the PEG content was less than or equal to 58% were very small. Below this value the PPF concentration was too high for the copolymer to form many crystallites. As the PEG concentration increased, the melting peaks increased in temperature and narrowed, which indicates that larger and more uniform crystallites were being formed. The changes in the melting temperature due to changes in the reaction design parameters given in Table 4.1 indicate that the only significant increases in the melting temperature were caused by increasing either the molecular weight or the percentage of the PEG component. A similar analysis on the effects of the design parameters on the percent crystalline PEG again indicates that the most significant increases are achieved by increasing either the molecular weight or the weight percent of PEG (data not shown).

The glass transition temperatures of PPF were -31.35 and -20.44°C for the low and high molecular weight polymers, respectively. The glass transition temperature for PEG was not evident even when the scanning run was initiated at -150°C, however, the literature value for PEG of nominal molecular weight 2,000 is -60°C (Rashkov et al., 1996). A single glass transition temperature was observed in all copolymer formulations between -42.24 and -54.14 °C. The presence of a single T_g between that of the starting homopolymers suggests the absence of microphase separation for all formulations of the copolymer (Krause, 1978).
Figure 4.7. DEPT-135 $^{13}$C NMR spectra of copolymer 4, made from PEG with $\overline{M}_w=4,570$ in CDCl$_3$. 
Figure 4.8. DEPT-135 $^{13}$C NMR spectra of PEG-diacrylate with $\bar{M}_n$=500 in CDCl$_3$. 
<table>
<thead>
<tr>
<th>Polymer</th>
<th>$T_m$ onset (°C)</th>
<th>$T_m$ max. (°C)</th>
<th>$T_g$ onset (°C)</th>
<th>$T_g$ midpt. (°C)</th>
<th>$DH_f$ (cal/g)</th>
<th>Percent Crystalline PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 2K</td>
<td>47.8 ± 0.4</td>
<td>52.4 ± 0.1</td>
<td>—</td>
<td>—</td>
<td>39.1 ± 0.9</td>
<td>—</td>
</tr>
<tr>
<td>PEG 4.6K</td>
<td>54.8 ± 0.2</td>
<td>58.6 ± 0.4</td>
<td>—</td>
<td>—</td>
<td>41.2 ± 1.0</td>
<td>—</td>
</tr>
<tr>
<td>PPF 5 hours</td>
<td>—</td>
<td>—</td>
<td>-31.4 ± 1.9</td>
<td>-27.0 ± 1.8</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PPF 10 hours</td>
<td>—</td>
<td>—</td>
<td>-20.4 ± 0.1</td>
<td>-14.8 ± 0.2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Copolymer 1</td>
<td>26.5 ± 0.3</td>
<td>34.6 ± 0.3</td>
<td>-54.1 ± 0.2</td>
<td>-51.4 ± 0.1</td>
<td>17.5 ± 0.7</td>
<td>13.5</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>24.2 ± 0.6</td>
<td>29.7 ± 0.1</td>
<td>-42.2 ± 0.1</td>
<td>-37.7 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Copolymer 3</td>
<td>25.0 ± 0.8</td>
<td>28.4 ± 0.8</td>
<td>-43.5 ± 1.3</td>
<td>-38.2 ± 1.2</td>
<td>0.2 ± 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Copolymer 4</td>
<td>28.0 ± 1.7</td>
<td>36.0 ± 0.8</td>
<td>-43.8 ± 2.2</td>
<td>-36.1 ± 2.8</td>
<td>15.4 ± 0.5</td>
<td>12.4</td>
</tr>
<tr>
<td>Copolymer 5</td>
<td>27.7 ± 1.4</td>
<td>36.7 ± 0.5</td>
<td>-46.1 ± 0.5</td>
<td>-41.9 ± 1.1</td>
<td>9.9 ± 0.2</td>
<td>12.8</td>
</tr>
<tr>
<td>Copolymer 6</td>
<td>37.7 ± 0.5</td>
<td>45.2 ± 0.2</td>
<td>-46.1 ± 0.8</td>
<td>-41.3 ± 0.6</td>
<td>21.2 ± 0.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Copolymer 7</td>
<td>39.7 ± 0.6</td>
<td>46.4 ± 0.7</td>
<td>-44.6 ± 3.4</td>
<td>-38.7 ± 4.9</td>
<td>20.1 ± 0.3</td>
<td>15.0</td>
</tr>
<tr>
<td>Copolymer 8</td>
<td>30.4 ± 0.9</td>
<td>38.8 ± 0.6</td>
<td>-43.7 ± 1.1</td>
<td>-38.1 ± 0.7</td>
<td>8.8 ± 0.3</td>
<td>14.0</td>
</tr>
</tbody>
</table>

Table 4.3. Thermal properties of P(PF-co-EG) copolymers.
4.3.c Solubility Properties

The solubility data demonstrated that the copolymer formulations were highly amphiphilic. Both molecular weights of PEG examined were insoluble in ethyl ether and cyclohexane, but the high molecular weight PEG was also insoluble in acetone, toluene, DMF, and THF. The low molecular weight formulation of PPF was insoluble in water and cyclohexane, while the high molecular weight, which had been reacted for ten hours, was insoluble in water, methanol, and cyclohexane. The resulting copolymers, however, were soluble in water as well as all organic solvents with the exception of ethyl ether and cyclohexane. Thus, the copolymer retained the good solubility characteristics of both the parent homopolymers.

4.4 Conclusions

The terminal hydroxyl groups of poly(ethylene glycol) were reacted with poly(propylene fumarate) in a transesterification reaction to form an ester linkage between the two homopolymers. Several different copolymer formulations were investigated and their molecular weights and compositions were determined. It was found that this reaction scheme produced a copolymer consisting primarily of diblock and triblock forms with the average PEG block number between 0.9 and 1.6. These copolymers all showed a melting peak and a single glass transition temperature, and there was no evidence of microphase separation. In addition, these copolymers showed enhanced solubilities compared to the parent homopolymers.
CHAPTER FIVE

PREPARATION AND CHARACTERIZATION OF POLY(PROPYLENE FUMARATE-CO-ETHYLENE GLYCOL) HYDROGELS

5.1 Introduction

The present chapter focuses on the evaluation of the swelling behavior and mechanical properties of P(PF-co-EG) hydrogels. Eight different copolymer compositions were investigated in order to evaluate the effects of four different design parameters: PEG molecular weight, PPF molecular weight, copolymer reaction time, and ratio of PEG to PPF. Two different crosslinking formulations were also tested, one with a crosslinking monomer and one without. We wanted to determine the crosslinking density, the degree of swelling in aqueous solution, the tensile properties, as well as the dynamic mechanical properties of the crosslinked copolymer. We also examined how these properties could be altered in order to optimize this material for its intended use.

5.2 Materials and Methods

5.2.a Polymer Synthesis

The synthesis of PPF has been previously described (Peter et al., 1997c). Briefly, fumaryl chloride (Aldrich, Milwaukee, WI) was added dropwise to a three fold molar excess of propylene glycol (Acros, Pittsburgh, PA) at room temperature in the absence of any catalyst. HCl was given off as a gas and trapped in aqueous NaOH. The resulting intermediate fumaric diester was then purified by solution-precipitation in chloroform and petroleum ether, respectively. The second step was a transesterification of the intermediate
to form the linear polyester, PPF. Finally, the copolymer was formed by adding PEG (Aldrich, Milwaukee, WI) to the reaction vessel at 160°C under a vacuum of approximately 115 mm Hg (Suggs et al., 1997b). Antimony trioxide was added as a basic catalyst and propylene glycol was removed by condensation. The entire reaction was performed under nitrogen by the use of a sparging apparatus within the reaction vessel. The resulting copolymer was again purified by solution-precipitation as described above. Excess chloroform and petroleum ether were removed by roto-evaporation. Gel permeation chromatography (GPC) was previously performed on the eight different copolymer formulations in order to determine molecular weights and compositions (Suggs et al., 1997b).

5.2.b Fabrication of Crosslinked Copolymers

Crosslinked copolymer networks were fabricated with two different formulations. The first was made using a vinyl monomer, and the second was made without this agent in order to examine the crosslinked copolymer alone. The proposed reaction scheme with the crosslinking monomer, N-vinyl pyrrolidinone (VP) is given in Figure 5.1. In the first formulation, we used VP at a 1:1 ratio of copolymer to monomer (wt/vol), 20:1 ratio of copolymer to benzoyl peroxide (BP) initiator (wt/wt), and catalytic amounts (1-5 µL/g of copolymer) of N,N-dimethyl-p-toluidine (DMT) at 25°C. This formulation was allowed to crosslink overnight prior to testing. The second formulation consisted of a 10:1 ratio of copolymer to BP (wt/wt) in enough chloroform to dissolve the mixture and was dried by roto-evaporation. This formulation was then heated at 60°C overnight to crosslink. Heat was used to accelerate the peroxide cleavage in the second formulation due to the insolubility of DMT in the dry copolymer.
5.2.c Swelling Studies

Films of copolymer formulations made without VP were crosslinked in 5 cm diameter glass Petri dishes with Teflon-coated bottoms (Bytac, Akron, OH). These crosslinked samples were 2-3 mm thick depending on the exact amount of copolymer used. They were vacuum-dried at 25°C and 10 μm Hg overnight prior to use to remove any residual solvents. They were then weighed, and their volumes were measured by displacement in a nonsolvent, cyclohexane (Aldrich, Milwaukee, WI). This was done by suspending the sample on a hook attached to a hanging pan balance. The nonsolvent was contained in a beaker which did not touch any part of the balance apparatus. Weight and volume measurements were taken after soaking the samples in distilled deionized H₂O (ddH₂O) and again after vacuum-drying until no weight change was observed. In all cases, the films were fully hydrated within one hour.
Figure 5.1. One proposed reaction scheme for the crosslinking of the diblock form of P(PF-co-EG) beginning with a poly(N-vinyl pyrrolidinone) living chain which has been initiated by the radical initiator, benzoyl peroxide. Initiation and propagation can also occur with double bonds on P(PF-co-EG) as in the case of crosslinking without VP.
Using procedures outlined in Peppas and Barr-Howell (Peppas and Barr-Howell, 1988), the swelling data were used to determine the ratio of the final dry weight of the polymer sample after swelling and vacuum-drying to the initial sample weight, gel fraction; the equilibrium volume swelling ratio, $Q$; as well as the molecular weight between crosslinks, $\overline{M}_c$. In this case where crosslinks were introduced without the presence of a solvent, the Flory and Rehner equilibrium swelling model was appropriate (Peppas and Barr-Howell, 1988):

$$\frac{1}{\overline{M}_c} = \frac{2}{\overline{M}_n} \left( \frac{\overline{v}}{V_1} \right) \left[ \ln(1 - u_{2,s}) + u_{2,s} + \chi_1 u_{2,s}^2 \right] \frac{u_{2,s}^3}{u_{2,s}^3 - v_{2,s}/2}$$  (5.1)

Here, $\overline{M}_n$ is the number average molecular weight of the linear polymer, $\overline{v}$ is the specific volume of the polymer, $V_1$ is the molar volume of the swelling agent and $\chi_1$ is the Flory-Huggins polymer-solvent interaction parameter. The equilibrium polymer volume fraction, $u_{2,s}$, was calculated as shown in equation (3) from the following measured weights.

The first measured weight was the initial weight of the crosslinked sample in air, $w_{a,r}$, which was measured after crosslinking and vacuum-drying. The sample was placed in cyclohexane and the initial weight of the crosslinked sample in a nonsolvent, $w_{n,r}$, was measured. The sample was then placed in distilled, deionized water and swollen to equilibrium and then removed to determine $w_{a,s}$, the weight in air of the sample at equilibrium swelling. The sample was then placed in cyclohexane again to get $w_{n,s}$, the weight in a nonsolvent of the sample at equilibrium swelling. Finally, the sample was vacuum-dried and the final dry weight of the sample gel after swelling, $w_{a,d}$, was measured. The density of the polymer, $\rho_p$, was calculated from the displacement of nonsolvent, given its density, $\rho_n$, which for cyclohexane is 0.79 g/cm³.
\[ \rho_p = \frac{w_{a.r}}{w_{a.r} - w_{a.r}} \rho_n \quad (5.2) \]

Then the equilibrium polymer volume fraction was calculated using \( V_p = \frac{w_{a.d}}{\rho_p} \) and \( V_{g,s} = \frac{w_{a.s} - w_{a.s}}{\rho_n} \) from the following equation:

\[ v_{s,s} = \frac{V_p}{V_{g,s}} \quad (5.3) \]

Given the other parameters, such as the polymer molecular weights presented in Table 5.1 (from GPC analysis) and the molar volume of the solvent (the value for water is 18.152 cm\(^3\)/mol), the crosslinking density was calculated from an estimate of the Flory-Huggins polymer-solvent interaction parameter, \( \chi_1 \), for each of the copolymers. The values of \( \chi_1 \) for pure PEG of nominal molecular weight 5,000 were used. These values were 0.5 for copolymer numbers 1, 4, 6, and 7; 0.6 for copolymers 2 and 5; and 0.7 for copolymers 3 and 8 and depended on the volume fraction of polymer in the solvent (Barton, 1990).
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Molecular Weight, $\overline{M}_n$</th>
<th>Ultimate Tensile Stress, $s_b$ (MPa)</th>
<th>Tensile Modulus, E (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copolymer 1</td>
<td>8200 ± 150</td>
<td>0.23±0.09</td>
<td>2.16±0.34</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>7370 ± 10</td>
<td>0.82±0.37</td>
<td>6.14±2.04</td>
</tr>
<tr>
<td>Copolymer 3</td>
<td>8050 ± 20</td>
<td>1.06±0.13</td>
<td>11.02±1.00</td>
</tr>
<tr>
<td>Copolymer 4</td>
<td>10060 ± 90</td>
<td>0.56±0.15</td>
<td>4.62±0.77</td>
</tr>
<tr>
<td>Copolymer 5</td>
<td>13090 ± 20</td>
<td>0.91±0.08</td>
<td>5.05±1.36</td>
</tr>
<tr>
<td>Copolymer 6</td>
<td>13950 ± 40</td>
<td>0.15±0.03</td>
<td>1.11±0.20</td>
</tr>
<tr>
<td>Copolymer 7</td>
<td>14200 ± 70</td>
<td>0.32±0.14</td>
<td>1.90±0.67</td>
</tr>
<tr>
<td>Copolymer 8</td>
<td>14080 ± 160</td>
<td>1.44±0.06</td>
<td>20.66±2.42</td>
</tr>
</tbody>
</table>

Table 5.1. Number average molecular weights of eight copolymer formulations as previously determined by gel permeation chromatography\(^2\) and their corresponding tensile testing data.
5.2.d Dynamic Mechanical Testing

Copolymer films were made both with and without VP by injection-molding, and cut into 1cm x 2cm x 1mm films for testing. The injection molding system consisted of 6 inch long, 20 gauge needle mounted on a syringe and inserted into a mold. The cavity in the Teflon mold was machined to 1mm depth and, the mold was then clamped to a glass plate in order to visualize the film formation. A dynamic mechanical analyzer (DMA) (model 983, TA Instruments, Newark, DE) was used to determine complex dynamic flexural moduli, $|E^*|$, and complex dynamic shear moduli, $|G^*|$. The samples were swollen first overnight in ethanol and then in ddH$_2$O to avoid cracking. The samples were equilibrium swollen and run at 25°C, at a frequency of 1 Hz, and at a strain of approximately 0.1%. The testing was performed in the flexural mode and lasted 15 min. The value of $\bar{M}_c$ was also determined from the shear modulus, $|G^*|$, an approximation for $G$, using the following equation derived from rubber elasticity theory (Anseth et al., 1996):

$$\frac{1}{\bar{M}_c} = \frac{2}{\bar{M}_n} + \frac{G}{RT\rho}$$  \hspace{1cm} (5.4)

Here, R is the ideal gas constant, T is the temperature in Kelvin, and $\rho$ is the density of the wet hydrogel as determined by swelling studies.

5.2.e Tensile Testing

Tensile tests were performed on a Vitrodyne 1000 Universal Materials Tester (Chattillon, Greensboro, NC) at a crosshead speed of 0.1 mm/sec. The crosslinked copolymers were made with VP and injection-molded into dogbone shapes which were 114 mm in total length, 33 mm in length at the narrow section, 6 mm wide at the narrowest
point, and 1 mm thick. They conformed to ASTM standard D639-91 for testing tensile properties of plastics. The samples were swollen first overnight in ethanol and then in ddH₂O until equilibrium was reached, and they were subsequently tested to failure. Stress and strain were calculated based on swollen dimensions as calculated from the swelling ratio. Tensile modulus was then determined from the slope of the linear region of the stress/strain graph. The samples were marked 20 mm from each end prior to swelling and then clamped even with the markings after hydration. The swelling ratio was determined from the Vitrodyne output based on the distance between marks both before and after swelling.

5.2.f Statistical Design

Several parameters were varied in order to assess their effects on the properties of the copolymer. This was accomplished through the use of a resolution IV, two level, fractional factorial design (Box et al., 1978). The design is given in Table 5.2. The parameters were varied as follows: PEG nominal molecular weight was either 2,000 or 4,600, transesterification time for the PPF homopolymer was either 5 or 10 hours, copolymer transesterification time was either 5 or 10 hours, and weight percent PEG was either 33% or 66%. For a transesterification reaction, molecular weight of the linear chain increases with increasing reaction time. In total, eight copolymers were made and three repetitions of each of the tests were performed except for the tensile tests in which five samples of each copolymer formulation were tested. Statistical comparisons were made using a student's t-test with a 95% confidence interval.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Nominal Molecular Weight of PEG</th>
<th>PPF Reaction Time</th>
<th>Copolymer Reaction Time</th>
<th>% PEG by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copolymer 1</td>
<td>2,000</td>
<td>5 hours</td>
<td>5 hours</td>
<td>66</td>
</tr>
<tr>
<td>Copolymer 2</td>
<td>2,000</td>
<td>5 hours</td>
<td>10 hours</td>
<td>33</td>
</tr>
<tr>
<td>Copolymer 3</td>
<td>2,000</td>
<td>10 hours</td>
<td>5 hours</td>
<td>33</td>
</tr>
<tr>
<td>Copolymer 4</td>
<td>2,000</td>
<td>10 hours</td>
<td>10 hours</td>
<td>66</td>
</tr>
<tr>
<td>Copolymer 5</td>
<td>4,600</td>
<td>5 hours</td>
<td>5 hours</td>
<td>33</td>
</tr>
<tr>
<td>Copolymer 6</td>
<td>4,600</td>
<td>5 hours</td>
<td>10 hours</td>
<td>66</td>
</tr>
<tr>
<td>Copolymer 7</td>
<td>4,600</td>
<td>10 hours</td>
<td>5 hours</td>
<td>66</td>
</tr>
<tr>
<td>Copolymer 8</td>
<td>4,600</td>
<td>10 hours</td>
<td>10 hours</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 5.2. Resolution IV fractional factorial design showing the eight combinations of high and low values for four different design parameters.
5.3 Results and Discussion

5.3.a Swelling Studies

The gel fraction was calculated from the swelling properties of the copolymer. This value is a ratio of the final dry weight of the polymer sample after equilibrium swelling and vacuum-drying to the initial sample weight and is a measure of the degree to which the crosslinking reaction has proceeded. The gel fractions ranged from $0.78 \pm 0.05$ to $0.86 \pm 0.01$, which show that the reaction is relatively efficient for all copolymer formulations. The equilibrium volume swelling ratio, $Q$, which is a ratio of the equilibrium swollen volume to the initial sample volume, varied from $1.5 \pm 0.1$ to $3.0 \pm 0.1$. This is typical for moderately swollen hydrogels (Peppas and Barr-Howell, 1988). Figure 5.2 shows the effects of the design parameters on the resulting value of $Q$. Increasing the weight percent PEG in the copolymer increases the swelling ratio, while increasing the molecular weight of PPF decreases the ratio.

The approximation of the Flory-Huggins polymer-solvent interaction parameter as described above leads to values of $\bar{M}_e$ which range from $300 \pm 120$ to $1190 \pm 320$ (Figure 5.3). A reasonable number would fall between about 156 (the minimum possible distance between unsaturated sites in pure PPF) and the molecular weight of a single chain of PPF homopolymer, about 500-1300. The relatively low values of $\bar{M}_e$ suggest that the PEG chains are not involved in the crosslinked section of the molecule. This case is supported by the effects of the design parameters on the swelling ratio. With a triblock copolymer of which only the central block is crosslinkable, the primary mode of affecting the crosslinking density would be to change either the molecular weight or the weight percent of the crosslinkable group as described above. As the crosslinking density is increased, the hydrogel is less permeable to water and the degree of swelling is decreased.
Figure 5.2. Magnitude of the change in the equilibrium swelling ratio (Q) of the copolymers caused by increasing the values of the four different design parameters. Error bars stand for means ± standard error of the effect. (n=3, standard error=0.06)
5.3.b Dynamic Mechanical Analysis

From DMA measurements, the calculated values for $\bar{M}_c$ range from 630±160 to 3150±480. These values are also shown in Figure 5.3 and are consistently higher than those determined by swelling studies. However, only copolymers 1 and 5 gave statistically different values for $\bar{M}_c$ as determined by the two methods. One explanation for this difference may be that the estimation of $\chi_1$ based on pure PEG fails for certain PEG copolymer compositions. In an effort to develop an experimentally determined value of $\chi_1$ for P(PF-co-EG) hydrogels we have plotted $\chi_1$ as determined by DMA versus $v_{2,3}$ as determined by swelling studies. The data were curve-fitted as shown in Figure 5.4 and gave the following equation:

$$\chi_1 = 0.232 + 1.016 \, v_{2,3}$$  (5.5)
Figure 5.3. Molecular weight between crosslinks for eight different copolymer formulations as determined by both swelling studies and DMA. Dotted bars represent data from swelling studies and hash marked bars represent data from DMA. Error bars stand for means ± standard deviation. (n=3. * designates significant difference at 95% confidence limit)
Figure 5.4. Scatter plot of $\chi_1$ as determined by DMA versus $\nu_{2,s}$ as determined by swelling. (correlation coefficient, $r^2=0.847$)
The complex dynamic flexural moduli, $|E^*|$, were also determined by DMA on copolymer formulations fabricated both with and without VP. The values for $|E^*|$ ranged from $0.9 \pm 0.2$ MPa to $13.1 \pm 1.1$ MPa for the formulations with VP and $1.6 \pm 0.5$ MPa to $13.9 \pm 3.4$ MPa for the formulations without VP. These data, along with the values for $|G^*|$ are given in Table 5.3. In comparison, the value for dynamic tensile modulus of canine aorta has been measured at $1.55$ MPa under the same strain at the same frequency and that of elastin, the primary component of the aortic wall, has been measured at $1.90$ MPa (Apter and Marquez, 1968). Figure 5.5 shows the effects of the design parameters on the resulting values of $|E^*|$. Increasing the molecular weight of PPF or the molecular weight of PEG increases the elastic moduli and therefore reduces hydrogel compliance. Conversely, increasing the weight percent of PEG decreases the $|E^*|$ and increases compliance. This result is again due to the fact that the PPF is the only crosslinkable group. As the relative amount of PPF is increased, more crosslinks are introduced, and consequently, the material becomes less compliant. Therefore, in order to minimize $|E^*|$ in the resulting crosslinked material, the molecular weight of PPF as well as the molecular weight of PEG should be low and the relative weight percent of PEG should be high. However, it has also been shown that a reduction in thrombogenicity by the presence of covalently bound PEG only occurs for high molecular weights between approximately 10,000 and 20,000 (Merrill, 1992).
| Sample Name | Flexural modulus, $|E'|$ w/ VP (MPa) | Flexural modulus, $|E'|$ w/o VP (MPa) | Shear modulus, $|G'|$ w/ VP (MPa) | Shear modulus, $|G'|$ w/o VP (MPa) |
|-------------|----------------------------------|----------------------------------|---------------------------------|---------------------------------|
| Copolymer 1  | 0.87±0.19                        | 2.13±0.19                        | 0.30±0.06                       | 0.73±0.06                       |
| Copolymer 2  | 2.99±0.32                        | 3.21±0.95                        | 1.00±0.07                       | 1.10±0.34                       |
| Copolymer 3  | 1.69±1.43                        | 7.32±2.61                        | 1.95±0.50                       | 0.99±0.76                       |
| Copolymer 4  | 5.64±0.18                        | 2.29±1.26                        | 0.59±0.07                       | 2.53±0.89                       |
| Copolymer 5  | 2.39±0.12                        | 1.57±0.47                        | 0.83±0.04                       | 0.55±0.16                       |
| Copolymer 6  | 2.65±0.12                        | 6.65±1.43                        | 0.92±0.04                       | 2.32±0.50                       |
| Copolymer 7  | 3.87±0.39                        | 11.15±0.95                       | 1.35±0.13                       | 2.16±0.33                       |
| Copolymer 8  | 13.14±1.13                       | 13.88±3.39                       | 4.60±0.40                       | 4.85±1.21                       |

Table 5.3. Flexural and shear moduli as determined by DMA for all formulations of the copolymer made both with and without N-vinyl pyrrolidinone.
Figure 5.5. Magnitude of the change in complex dynamic flexural modulus, $|E'|$, of the copolymers made with VP caused by increasing the values of the four different design parameters. Error bars stand for means ± standard error of the effect. (n=3, standard error=0.28)
5.3.c Tensile Testing

The ultimate tensile stress or stress at break, $s_b$, on the formulations made with VP ranged from 0.15\pm 0.03 \text{ MPa} to 1.44\pm 1.06 \text{ MPa} at typical strains between 10 and 20\%. Tensile modulus, $E$, ranged from 1.11\pm 0.20 \text{ MPa} to 20.66\pm 2.42 \text{ MPa} (Table 5.1). In comparison, these values are significantly lower than those for PEG copolymers with polyurethanes (Silver et al., 1994) or poly(ethylene terephthalate) (Gilding and Reed, 1979). However, they are comparable to PEG copolymers with poly(lactic acid) for which ultimate tensile strengths have been reported in the range of 4-7 MPa (Cohn and Younes, 1988). The effects of the four design parameters on the resulting values of $s_b$ and $E$ are shown in Figures 5.6 and 5.7. Both stress and modulus increase with increasing PPF molecular weight and decrease with increasing PEG content. The molecular weight of PEG as well as the copolymer reaction time have little effect on ultimate tensile stress or $E$. This again shows that changing the crosslinking density by varying the size and amount of PPF is the primary method of affecting the mechanical properties.
Figure 5.6. Magnitude of the change in the ultimate tensile stress, $s_b$, of the copolymers made with VP caused by increasing the values of the four different design parameters. Error bars stand for means ± standard error of the effect. (n=5, standard error=0.07)
Figure 5.7. Magnitude of the change in the tensile modulus, $E$, of the copolymers made with VP caused by increasing the values of the four different design parameters. Error bars stand for means ± standard error of the effect. ($n=5$, standard error=0.54)
5.4 Conclusions

The equilibrium volume swelling ratios of P(PF-co-EG) block copolymer hydrogels are characteristic of moderately swollen hydrogels, and, along with the relatively low values of $M_c$, show that the hydrogel is highly crosslinked. The swelling ratio can be reduced by increasing the relative amount or molecular weight of the PPF block. Copolymer hydrogels made both with and without VP showed similar values for flexural moduli and these values are comparable to those of canine aorta. The moduli can be decreased by increasing the relative amount of PEG or decreasing its molecular weight or the molecular weight of PPF. The ultimate tensile stresses on the hydrogels made with VP were relatively low compared to other PEG copolymers, however, these values can be increased by increasing the relative amount or molecular weight of PPF.

Many applications in tissue engineering require a material which has the biocompatibility and drug delivery potential of a hydrogel. Hydrogels, however, typically have very poor mechanical properties. We have designed a copolymer with relatively high mechanical properties due to the presence of crosslinkable groups along the molecular backbone. We have developed this material for use in the vasculature to protect the vessel wall against restenosis as well as potentially serving as a carrier for bioactive molecules. We envision that this material could be optimized for use in other applications and have attempted to give a method for altering physical properties by changing design parameters.
CHAPTER SIX

IN VITRO AND IN VIVO DEGRADATION OF POLY(PROPYLENE FUMARATE-CO-ETHYLENE GLYCOL) HYDROGELS

6.1 Introduction

In this chapter, we investigated the degradation behavior of P(PF-co-EG) both in vitro and in vivo. The composition of the copolymer as well as the molecular weight of PEG were varied in order to assess their effects on degradation mode, time of degradation, and extent of inflammatory response. Mechanical properties, dimensional changes, weight loss, and morphological changes were examined in vitro. In a subcutaneous rat model, analysis of these changes along with histological evaluation were compared with the in vitro model.

6.2 Materials and Methods

6.2.a Copolymer Synthesis

PPF was produced as previously described (Peter et al., 1997c). Briefly, fumaryl chloride (Aldrich, Milwaukee, WI) was added slowly to an excess of propylene glycol (Acros, Pittsburgh, PA) at 25°C under nitrogen. This resulted in the formation of an intermediate diester. The PPF was then formed in a transesterification reaction of the intermediate at 160°C under a vacuum of approximately 115mmHg using antimony trioxide (Aldrich) as a catalyst. Lastly, PEG (Aldrich) was added to the reaction under the same conditions to form the copolymer (Suggs et al., 1997b). The resulting copolymer was purified by solution-precipitation using chloroform and petroleum ether (Fisher,
Pittsburgh, PA). Roto-evaporation was used to remove excess solvents. Three copolymer formulations were fabricated as shown in Table 6.1. The first was a base formulation, the second had an increased weight percent PEG, and the third had a higher molecular weight PEG. Gel permeation chromatography (GPC) was performed on the copolymer formulations in order to determine molecular weight distributions using a differential refractometer (Waters, Model 410, Milford, MA). Polystyrene standards were used to obtain a universal calibration curve, and the resulting molecular weights are also given in Table 6.1.

6.2.b Fabrication of Copolymer Hydrogels

Copolymer hydrogels were fabricated at 25°C using a vinyl monomer, N-vinyl pyrrolidinone (VP) (Aldrich), at a 1:1 ratio of copolymer to monomer (wt/vol), 20:1 ratio of copolymer to initiator, benzoyl peroxide (BP) (Aldrich) (wt/wt), and 1 µL of N,N-dimethyl-p-toluidine (DMT) (Aldrich) per gram of copolymer. The samples were made using an injection-molding system consisting of a 6in, 20G needle inserted into a Teflon mold which had been machined to 1mm depth and clamped to a glass plate. They were cut either into 1cm x 2cm rectangles or injection-molded directly into dogbone shapes, which were 114mm in total length, 33mm in length at the narrow section, 6mm wide at the narrowest point, and 1mm thick, according to ASTM standard D639-91. The samples were then weighed dry and swollen first overnight in ethanol to remove the uncrosslinked (sol) fraction and then placed in phosphate-buffered saline (PBS), pH 7.4, for eight hours. The exact dimensions of the swollen films were then measured prior to use.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>PEG/PPF</th>
<th>PEG $M_w$</th>
<th>Copolymer $M_w$</th>
<th>Copolymer $M_n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copolymer 25/5K</td>
<td>25/75</td>
<td>4,600</td>
<td>12,800</td>
<td>10,400</td>
</tr>
<tr>
<td>Copolymer 50/5K</td>
<td>50/50</td>
<td>4,600</td>
<td>14,500</td>
<td>11,200</td>
</tr>
<tr>
<td>Copolymer 25/10K</td>
<td>25/75</td>
<td>10,500</td>
<td>18,200</td>
<td>15,500</td>
</tr>
</tbody>
</table>

Table 6.1. Compositions by weight and molecular weights of the three different copolymer formulations used in this study.
6.2.c In Vitro Degradation

Swollen 1cm x 2cm x 1mm films were placed in 40ml of PBS at 37°C on a rotating shaker. Dogbones were placed in 250ml of PBS under the same conditions. The PBS was changed for all the samples every 8 hours for the first day, every day for the first week, and weekly thereafter in order to keep the pH relatively constant. Copolymer films were taken out at 1 day, and at 1, 3, 6, 9, and 12 weeks. The swollen dimensions of the films were measured. The films were tested using dynamic mechanical analysis (DMA), dehydrated into ethanol, and dried using an Electron Microscopy Sciences (Fort Washington, PA) critical-point drier. The resulting dry weights were recorded. The dogbones were tested to failure after 8 hours and at 3 and 12 weeks.

6.2.d Dynamic Mechanical Analysis

Complex dynamic flexural modulus was determined using a DMA (Model 983, TA Instruments, Newark, DE). The swollen DMA samples were first coated with vacuum grease in order to avoid any water loss during the testing. They were then run at a frequency of 1 Hz, and at a strain of approximately 0.1%. The testing was performed at 37°C and lasted for approximately 15 min. Three samples were tested for each time point.

6.2.e Tensile Testing

A Vitrodyne 1000 Universal Materials Tester (Chattillon, Greensboro, NC) was used to determine elastic modulus and ultimate tensile stress. The swollen samples were tested to failure at a crosshead speed of 0.1 mm/sec. Stress and strain were calculated based on swollen dimensions. The samples were all mounted in the same relative position taking
into account any differences in swelling. Stress/strain graphs were then calculated and used to determine tensile modulus. Five samples were tested for each time point.

6.2.f Scanning Electron Microscopy

The dried copolymer films were gold-coated with a Model 3 Pelco Sputter Coater 91000 (Reading, CA). Images were taken from a JEOL JSM-5300 (Boston, MA) with an accelerating voltage of 25V at 75X magnification. Copolymer films were positioned at a 45° angle.

6.2.g In Vivo Degradation

Copolymer films of dimensions 1cm x 2cm x 1mm were implanted unilaterally on the backs of male Sprague-Dawley rats (300-350g). They were anesthetized intramuscularly with a cocktail consisting of 3.6mg/ml xylazine, 63.6mg/ml ketamine, 0.07mg/ml atropine at a concentration of 0.23ml/100 g body weight. An amount of 10mg of gentamycin was given at the time of surgery. The animals were prepared by shaving and applying a betadine solution. Two incisions were made unilaterally along the midportion of the back each about 2cm long. Pockets were formed subcutaneously around each incision, and the copolymer films were inserted. Each incision was closed with two, 4.0 ethilon sutures (Ethicon, Somerville, NJ). Animals were given food and water ad libidum and allowed free movement within their cages. Animals were euthanized with ethyl ether, and the samples were taken out at 1 day and 4 days, and at 1, 3, 6, 9, and 12 weeks. Three samples per time point were used for DMA, weight loss determination, and dimensional analysis. One sample per time point was used for histological analysis. Animal care protocols followed the NIH guidelines for the care and use of laboratory animals.
6.2.h Histological Analysis

After the animals were euthanized, the tissues surrounding the copolymer films were removed with the films intact whenever possible. These samples were fixed in 10% formalin for at least 48 hours. They were then serially dehydrated and paraffin-embedded prior to sectioning and staining with hematoxylin and eosin (H&E).

6.2.i Statistics

Statistical analysis was performed using an ANOVA test with a 95% confidence interval (p<0.05). Statistical significance between populations was determined from a student's unpaired t-test with a 95% confidence interval.

6.3 Results

6.3.a pH

The pH was maintained at a relatively constant value for the in vitro study as shown in Figure 6.1. There was a drop in pH after the PBS changing schedule went from daily to weekly, however, the numeric value never went below 7.1 for any of the copolymer formulations.
Figure 6.1. Change in pH over the 12 week time course for three different copolymer formulations (see Table 6.1). Error bars represent means±SD for n=3.
6.3.b Weight Loss

The majority of the weight loss for both the in vitro and in vivo samples occurred during the first week as shown in Figures 6.2a and 6.2b. At approximately 1 week for the in vitro study, the fraction of mass lost leveled off and was not significantly different for any formulation up to twelve weeks. For the in vivo study, copolymer 50/5K did not change after 1 week, while copolymers 25/5K and 25/10K decreased significantly over the time course. Copolymers 50/5K and 25/10K generally had the highest fraction of mass lost in both studies. At twelve weeks, copolymers 50/5K and 25/10K lost 56.9% and 59.0%, respectively, of their original mass in vitro, while copolymer 25/5K lost only 50.2%. In the in vivo study, copolymers 25/5K, 50/5K, and 25/10K were all very close at 61.9%, 58.6%, and 58.8%, respectively, of their initial mass lost at twelve weeks.

6.3.c Dimensional Changes

The normalized volume changes for both studies are given in Figures 6.3a and 6.3b. All of the copolymer formulations increased slightly in volume both in vitro and in vivo. Copolymer 25/5K increased by 0.8%, copolymer 50/5K by 0.4%, and copolymer 25/10K by 0.6%, in vitro over the 12 week study. For the in vivo study, copolymer 50/5K increased by 0.3%, which was very similar to the in vitro case, while copolymers 25/5K and 25/10K increased by 1.2% and 1.5%, respectively. These two were significantly greater in vivo than in vitro at twelve weeks.
Figure 6.2. Normalized change in copolymer film mass a.) *in vitro* and b.) *in vivo* for three different copolymer formulations (see Table 6.1). Error bars represent means ±SD for n=3.
Figure 6.3. Normalized volume change for three different formulations (see Table 6.1) of copolymer films degraded a.) in vitro and b.) in vivo. Error bars represent means ±SD for n=3.
6.3.4 Tensile Testing

Stress/strain curves were generated and representative curves for three different time points are shown in Figure 6.4. These curves showed the linear elastic behavior of the hydrogels. In addition, they showed that the stress at failure for a given strain decreased as the degradation time increased. This temporal decrease is shown for all three formulations in Figures 6.5a and 6.5b. Copolymers 25/5K and 25/10K decreased 80.4% and 80.2% in ultimate tensile stress after the first 3 weeks. The ultimate tensile stress for copolymer 50/5K, however, decreased by only 76.0%. The stress values for all three leveled out by 12 weeks at 0.31 MPa, 0.27 MPa, and 0.30 MPa, for copolymers 25/5K, 50/5K, and 25/10K, respectively. The tensile modulus showed similar but more pronounced trends with the values for copolymers 25/5K and 25/10K decreasing by 89.7% and 56.8% by three weeks. The value for copolymer 50/5K was significantly different from copolymers 25/5K and 25/10K for the first 3 weeks and decreased by 34.5%. Again, the tensile modulus values leveled off to values of 3.8MPa, 2.8 MPa, and 3.8 MPa for copolymers 25/5K, 50/5K, and 25/10K, respectively after 12 weeks.
Figure 6.4. Representative stress/strain curves of copolymer 25/5K at three different time points showing the linear elastic behavior of the hydrated films.
Figure 6.5. Variation in a.) tensile stress at failure and b.) tensile modulus for three different copolymer formulations (see Table 6.1) at three different time points. Error bars represent means ±SD for n=5.
6.3.e Dynamic Mechanical Analysis

Complex dynamic flexural moduli were determined by DMA on copolymer formulations degraded in vitro and in vivo, and these values are shown in Figures 6.6a and 6.6b. The in vitro values for complex flexural moduli were similar to those obtained by tensile testing. Copolymer 50/5K gave significantly different values than copolymers 25/5K and 25/10K during the first three weeks, decreasing by 32.5% compared to 72.9% and 83.0%. At twelve weeks, the modulus values were 1.30MPa, 0.48MPa, and 1.32MPa, for copolymers 25/5K, 50/5K, and 25/10K, respectively. For the in vivo case, the complex flexural modulus of copolymer 50/5K was statistically less than copolymers 25/5K and 25/10K for the first 3 weeks as well as showing the smallest decrease of 70.0%. Interestingly, the modulus of copolymer 25/5K was significantly less than that of copolymer 25/10K for weeks 1 and 3. Copolymers 25/5K and 25/10K showed decreases in complex flexural moduli of 89.6% and 74.7%, respectively. By six weeks, copolymer 50/5K did not have enough structural integrity to be tested, and no data could be collected for all three copolymers at nine and twelve weeks in vivo.

6.3.f Scanning Electron Microscopy

Representative SEMs of copolymer 25/5K are shown in Figure 6.7a and 6.7b. These micrographs showed only very slight morphological changes in the original cut edge of the copolymer film after 12 weeks of degradation in PBS. More pitting and irregularities was present in the 12 week sample.
Figure 6.6. Complex dynamic flexural modulus as determined from DMA a.) *in vitro* and b.) *in vivo* for three different copolymer formulations (see Table 6.1). Error bars represent means ±SD for n=3.
6.3.g Histology

At 24 hrs post-implantation, all three formulations exhibited an acute inflammatory response, characterized by cellular infiltration consisting primarily of neutrophils. There was evidence of bleeding as well as focal cellular necrosis. Copolymer 25/5K displayed an elevated acute response compared to the other formulations as shown by extensive neutrophilic infiltration. Figure 6.8a shows an H&E stained section of the tissue surrounding the 50/5K implant after four days. Fibroblasts were numerous and macrophages were also evident at this stage. Granulation tissue was present with newly formed capillaries. At three weeks, as shown in Figure 6.8b, the tissue displayed minimal chronic response with macrophages and a few giant cells present at the polymer/tissue interface. The fibrous capsule was compacted and consisted primarily of fibroblasts and collagen fibers. At six weeks, polymer fragments were evident embedded in the fibrous capsule. This phenomenon was more pronounced for copolymers 25/10K and 50/5K. Figure 6.8c shows a section at 12 weeks. The specimens displayed a mature fibrous capsule with a limited foreign body reaction at the implant interface. The ventral side of all the implants showed the majority of the particulate formation. There was evidence of biodegradation with small polymer particles being engulfed by macrophages, larger particles being engulfed by giant cells, and even larger particles remaining at the surface of the capsule.
Figure 6.7. Representative scanning electron micrographs of copolymer 25/5K taken at a.) 1 week and b.) 12 weeks *in vitro* (45° angle/75x magnification). The side view of the original cut edge shows only slight morphological changes.
Figure 6.8. H&E stained histological sections of the tissue surrounding copolymer formulation 50/5K at a.) 4 days, b.) 3 weeks, and c.) 12 weeks.
6.4 Discussion

The objective of this study was to provide an evaluation of the degradation behavior of a crosslinked PEG hydrogel as well as to examine the effect of copolymer composition and molecular weight on degradation both in vitro and in vivo. Specifically, the molecular weight of the PEG block and weight percent PEG were varied in the linear copolymer. In addition, the initial biocompatibility of this material was assessed over a 12 week time course in a subcutaneous rat model.

The PBS was changed frequently in the in vitro study to approximate a site which would not experience a build-up of low pH degradation or leachable products which could cause additional acid-catalyzed degradation (Chu, 1981). This was done in order to provide a controlled system against which to evaluate different effects.

The P(PF-co-EG) hydrogels degrade primarily by bulk degradation. Both the in vitro and in vivo studies showed the same mode of degradation. This was evidenced by the decrease in mechanical properties without concurrent decrease in dimensions. Polyesters such as these typically degrade in bulk, with an initial phase characterized by loss in mechanical strength followed by a second phase consisting of a decrease in mass (Suggs and Mikos, 1996). These materials typically do not exhibit the dimensional changes caused by surface erosion. There also did not appear to be dramatic morphological changes evident on the film surfaces.

Tensile testing was performed only on the in vitro samples due to the prohibitively large dimensions of the dogbones for the rat model. These data gave information which correlated well with DMA for the in vitro case and allowed inferences to be made with respect to tensile properties for the in vivo case. The copolymer samples showed some retention of tensile strength over the first three weeks, showing at least 20% of their original ultimate tensile stress. There was no significant difference between the tensile stress or modulus of copolymers 25/5K and 25/10K. This suggests that the PEG
molecular weight had no effect on tensile mechanical properties, which is consistent with a previous study (Suggs et al., in press). There was a significant difference, however, between the tensile modulus of copolymer 50/5K and the other two for the first three weeks. The values for 50/5K initially were very low in comparison, and copolymers 25/5K and 25/10K exhibited a large decrease in modulus over the time course. The PPF component of this copolymer was the only part of the structure which was crosslinkable as well the only one which was susceptible to ester hydrolysis. This indicates that increasing the weight percent of PPF not only increased the initial tensile modulus, but also increased the rate of decrease of the tensile modulus.

The complex dynamic flexural moduli correlated well with tensile moduli for the in vitro case. There were no significant differences between copolymers 25/5K and 25/10K over 12 weeks. Copolymer 50/5K, however, was significantly different from the other two for the first three weeks with all three leveling off at 6 weeks. The behavior of copolymer 50/5K was consistent in vitro and in vivo. For the 25% PEG copolymers, there were some differences in the degradation behavior. Copolymer 25/5K appeared to degrade at a faster rate in vitro than in vivo, while copolymer 25/10K appeared to degrade more slowly. However, copolymer 25/10K was only significantly different at week 1, and copolymer 25/5K was only significantly different at week 3.

Normalized weight loss over 12 weeks was similar for both studies. There was a decrease of 40-60% over the first week due to the leaching of the water-soluble uncrosslinked fraction. Afterwards, there was no significant change in weight loss for the in vitro case. For the in vivo case, only copolymers 25/5K and 25/10K showed significant decreases after 1 week. Copolymer 25/5K consistently lost less weight than the other two formulations. This was probably due to the extent of crosslinking which controls the mass of the sol fraction.

There were no losses in film volume for any of the copolymer formulations over 12 weeks for either study. In fact, all of the films increased in volume. This is cue to the fact
that when structural bonds are broken during degradation, this allows for the absorption of additional water into the polymer matrix. This swelling was less than 2% of the total volume for all copolymers at all time points. The in vitro and in vivo data correlated well until approximately 6 weeks. At this time there appeared to be relatively large increases in swelling for copolymers 25/5K and 25/10K. This, along with the dynamic mechanical and weight loss data suggested that the copolymers with the higher PPF weight percent degraded faster. There appeared to be no increase in the degradation rate due to increased hydrophilicity as might be expected. All of the copolymer formulations were highly swollen, and water uptake was not a limiting factor. The data also indicated that there was an increase in the rate of degradation for the in vivo case most probably due to a local decrease in pH. This can be caused by acidic degradation products or leachable components. The local pH around phagocytotic inflammatory cells can also be quite low due to the acidic contribution from lysosomes.

P(PF-co-EG) hydrogels showed good initial biocompatibility upon histological evaluation. The implantation elicited an acute inflammatory response characterized by infiltration of neutrophils. This was followed by development and maturation of a fibrous capsule around the implant up to the twelve week time point. This is a typical wound healing response to biomaterial implantation (Anderson et al., 1996). Copolymer 25/5K appeared to cause an increased acute response, however it also showed faster resolution and a more compact fibrous capsule. The 25/10K and 50/5K copolymers showed a minimal response at 24 hrs, although at twelve weeks the fibrous capsule surrounding these implants appeared less mature. This may be attributable to an increased formation of particulates in the latter two cases. The particulates caused the formation of a limited layer of granular tissue containing foamy macrophages and giant cells.
6.5 Conclusions

P(PF-co-EG) degrades primarily though bulk degradation both in vitro and in vivo. Significant weight loss due to degradation was observed only in the in vivo study in the case of the low weight percent PEG copolymers. There was no loss in film volume, however, there was a slight increase due to swelling as a result of hydrolytic degradation. Mechanical properties decreased dramatically during the first three weeks and leveled off until twelve weeks. Differences between mechanical properties in vitro and in vivo were evident only after six weeks. Increasing the weight percent of PEG in the copolymer decreased the rate of degradation. Increasing the molecular weight of PEG only affected the degradation by altering the extent of crosslinking of the starting hydrogel. P(PF-co-EG) hydrogels exhibited a normal inflammatory and wound healing response resulting in fibrous encapsulation with evidence of biodegradation at the biomaterial/tissue interface.
CHAPTER SEVEN

PLATELET ADHESION ON AN INJECTABLE, BIODEGRADABLE PEG COPOLYESTER UNDER BOTH STATIC AND FLOW CONDITIONS

7.1 Introduction

In this chapter, we investigated the adhesion and aggregation of platelets on P(PF-co-EG) hydrogels under both static and flow conditions. The effects of changes in the weight percent of the PEG component as well as the PEG molecular weight on the copolymer were examined. The relative extent of adhesion and aggregation were compared to reference materials of glass, silicone rubber, and crosslinked PPF homopolymer films. The morphology of platelets adherent to the test surfaces was examined using scanning electron microscopy (SEM) and environmental SEM (ESEM).

7.2 Materials and Methods

7.2.a Copolymer Synthesis

PPF was synthesized and characterized as previously described (Peter et al., submitted). Briefly, propylene glycol (Acros, Pittsburgh, PA) was mixed with potassium carbonate (Fisher, Pittsburgh, PA) in a 2:1 molar excess and dissolved in a double volume of chloroform to form a slurry. Fumaryl chloride (Aldrich, Milwaukee, WI) dissolved in an equal volume of chloroform (Aldrich) was then added slowly to the slurry in a molar ratio of 1:3, fumaryl chloride:propylene glycol. The reaction was performed at 25°C with vigorous stirring. The potassium carbonate was removed gravimetrically and the PPF was then formed in a transesterification reaction at 160°C under a vacuum pressure of
approximately 110 mmHg. PEG (Aldrich) was added to the reaction vessel under the same conditions to form the copolymer (Suggs et al., 1997c). The resulting copolymer was precipitated by solution-precipitation using chloroform and petroleum ether (Fisher). Roto-evaporation was used to remove excess solvents. Three different copolymer formulations were fabricated from the same PPF homopolymer. The first abbreviated as, 50/5K, consisted of 50% PEG by weight, with a number average molecular weight of 4,600. The second, 75/5K, consisted of 75% PEG by weight, and the third, 50/10K, consisted of PEG of molecular weight 10,500.

7.2.b Fabrication of Test Films

Glass coverslips (12 mm da. and 22x50 mm) were used as received (Fisher). Silicone rubber films were made from medical grade silicone (A-103, Factor II, Lakeside, AZ), and the initiator was incorporated at 10% w/w. The silicone was spread onto glass coverslips and allowed to crosslink overnight at 60°C. Copolymer hydrogel films were fabricated by first dissolving the copolymer in distilled deionized water (ddH₂O) at 50% w/v. The initiator, benzoyl peroxide (BP, Aldrich), was mixed with a vinyl monomer, N-vinyl pyrrolidinone (VP, Aldrich), at 33% v/v initiator in the monomer solution. The initiator and monomer were mixed with the copolymer solution at 50% w/v copolymer, and 1μL of N,N-dimethyl-p-toluidine (DMT) (Aldrich) was added. The resulting solution was poured onto a Teflon surface and a glass coverslip was placed over the hardening fluid to form a film. The films were allowed to crosslink overnight at room temperature. And they were then hydrated for 24 hrs in ddH₂O. The films were then cut either into 12 mm diameter films using a cork-borer or into rectangles of dimensions 22x50 mm. The PPF films were made in a similar fashion, the only difference being the exclusion of the aqueous component. All films were hydrated for 1 hr in phosphate-buffered saline (PBS) at pH 7.4 prior to testing.
7.2.c  Contact Angle Measurements

Water contact angles in air of the polymer surfaces were measured after equilibrium swelling in ddH₂O for 1-2 hrs. The measurements were taken using a contact angle goniometer (Rame-Hart, Mountain Lakes, NJ).

7.2.d  Platelet Suspension Preparation

Whole blood was drawn from healthy donors into a 1:6 volume of acid-citrate dextrose (65mM citric acid, 85 mM sodium citrate, 111 mM dextrose, pH 4.5). Washed platelets were prepared as described previously (Chow et al., 1992). Briefly, platelet-rich plasma (PRP) was prepared by centrifugation at 150xg for 15 min at 23°C. The plasma was drawn off and centrifuged further at 1000xg for 15 min at 23°C. This resulted in the formation of platelet-poor plasma (PPP) and a pellet. The pellet was washed and resuspended in a small amount of pH 7.0 HEPES buffer (10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄, 5.5 mM glucose, 3.5 g/L bovine serum albumin, 0.1 mM CaCl₂, 50 U/ml sodium heparin, and 2.5 U/ml apyrase, pH 7.0). The suspension was diluted to 10 ml in the low pH buffer and allowed to rest for 45 min. Afterwards, the platelets were centrifuged again at 1000xg for 10 min. The supernatant was decanted, and the pellet was then resuspended in a pH 7.4 HEPES buffer (10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgSO₄, 5.5 mM glucose, 3.5 g/L bovine serum albumin, 0.01 mM CaCl₂, pH 7.4). The platelet concentration was adjusted to 50x10⁶ cells/ml using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) prior to the adhesion assay.
7.2.e  

**Indium Oxine Assay**

For the following experiments, 10 mCi/ml of radio-labeled indium oxine was added during the platelet washing step and incubated for 10 min at 37°C (Thakur et al., 1976). Aliquots of 50 mL of both the supernatants and platelet suspensions were used to determine both the labeling efficiency and the specific activity of the suspension. A volume of 1 ml of platelet suspension was placed on each of the 12 mm diameter film surfaces in 24-well tissue culture plates. The films were incubated with the platelet suspension for 1 hr at 37°C. The films were then dip-rinsed twice in the pH 7.4 HEPES buffer. The adhesion of labeled platelets was quantified using a gamma counter.

7.2.f  

**Lactate Dehydrogenase (LDH) Assay**

Unlabeled platelet suspension (1 ml) was incubated on 12 mm diameter film surfaces as described for the radioactive assay (Tamada et al., 1995). After incubation, the films were dip-rinsed twice in the pH 7.4 HEPES buffer. They were then placed into 0.5% Triton-X100 for 1 hr at 25°C to lyse the adherent platelets. A volume of 1 ml of buffer was then removed, and the LDH activity was measured using an LDH endpoint concentration assay kit (cat. no. 500, Sigma Diagnostics). This activity was compared to lysed platelet suspensions of known concentrations in order to quantify the number of platelets on each film.

7.2.g  

**Flow Studies**

Whole blood was drawn from healthy donors into heparinized syringes at a concentration of 10U/ml blood. A parallel plate flow chamber was used in conjunction with a Harvard syringe pump as previously described (Lawrence et al., 1987). The test films
were mounted onto the chamber using a vacuum source with a silicone rubber gasket to separate the film from the chamber. Blood was passed over each film at a shear rate of 2000 s⁻¹ for 2 min. The films were then rinsed with PBS and placed either into a lysate buffer for LDH activity determination or a 2.5% gluteraldehyde solution for fixation.

7.2.h Scanning Electron Microscopy

Samples of each type of film were reserved for qualitative analysis via scanning electron microscopy. These films were fixed in 2.5% gluteraldehyde for 2 hrs immediately after the rinsing step. The glass and silicone rubber films were rinsed in PBS after fixation and serially dehydrated from water to 100% ethanol. They were then critical-point dried using a using an Electron Microscopy Sciences (Fort Washington, PA) critical-point drier. The dried glass and silicone films were coated with gold using a Model 3 Pelco Sputter Coater 91000 (Reading, CA). SEM images were taken in high vac mode from a Philips XL30 ESEM-FEG (Mahwah, New Jersey). The ESEM images from the polymer films were taken in wet mode without dehydration. This was done on cold stage at 5°C at a pressure of 5 torr in order to insure that the polymer morphology was maintained.

7.2.i Statistics

Statistical analysis was performed using a student's unpaired t-test with a 95% confidence interval (p<0.05). Eight repetitions were performed for the radio-labeled platelet assay, and three repetitions were performed for all other assays. All data are reported as mean±SD.
7.3 Results

7.3.a Contact Angle Measurements

All of the polymer surfaces demonstrated no advancing contact angles and were instantaneously wetted. This indicates that the polymers are very hydrophilic and have a theoretical contact angle of zero.

7.3.b Static Adhesion Studies

The number of platelets adhered to each of the film surfaces as determined by the \textsuperscript{111}indium oxine assay is shown graphically in Figure 7.1. PPF had the highest density of platelets adherent at 32.5±8.0 platelets/cm\textsuperscript{2}. Glass had an average value of 12.0±6.5 platelets/cm\textsuperscript{2}. Silicone rubber, and copolymer formulations 75/5K and 50/10K both showed significantly different values than glass for platelet surface coverage at 11.0±2.2, 10.6±4.0, and 12.4±5.7 platelets/cm\textsuperscript{2}, respectively. Formulation 50/5K, however, was not significantly different from glass, and there were no significant differences among any of the three copolymer formulations. In comparison, the results from the LDH assay were not statistically different from the results as determined from the \textsuperscript{111}indium oxine assay except in the case of PPF. The results from both assays are shown together in Figure 7.2.
Figure 7.1. Graph of platelet surface density on six different films as determined by the $^{111}$indium oxine assay. Error bars represent means ±SD for n=8. (* = statistically different than PPF, † = statistically different than glass)
Figure 7.2. Comparison graph of platelet surface density on six different films as determined by both the $^{111}$In-indium oxine assay and the LDH assay. Error bars represent means ±SD for $n=4$ for the LDH assay.
7.3.c Flow Studies

The number of adherent platelets on the test films treated with whole blood under flow conditions was quantified using the LDH assay. The results from these studies are shown graphically in Figure 7.3. The glass and silicone rubber films were not significantly different at surface coverages of 12.8±6.5 and 17.4±6.7 platelets/cm², respectively. The copolymer films, however, had dramatically fewer platelets on the surface. Copolymers 50/5K, 75/5K, and 50/10K had surface coverage values of 1.39±1.38, 0.07±0.08, and 1.00±0.50 platelets/cm², respectively.

7.3.d Platelet Morphology

Figure 7.4 shows micrographs of platelets that have adhered to both glass and silicone rubber films under static conditions. The formation of thrombi was evident on the glass coverslips. The platelets had formed large aggregates and the extension of pseudopodia was observed. In the case of the silicone rubber, however, the platelets were generally singular with the rounded shape typical of platelets in the inactivated state. Micrographs of films of PPF and copolymer 50/10K exposed to platelet suspensions are shown in Figure 7.5. A greater number of platelets are apparent on PPF films than the copolymers. The platelet morphology, however, is similar on all the polymer surfaces. The platelets appear singular and rounded, without evidence of thrombus formation. Figure 7.6 shows micrographs on glass under static and under flow. Again, thrombus formation is evident on the glass surface as well as the extension of pseudopodia. In comparison to the static assay, the platelets appear flattened and more circular. On the silicone rubber surfaces, even though there is no statistical difference in the number of platelets, the platelets do not appear aggregated. No adherent platelets were observed on the copolymer films tested under flow conditions.
Figure 7.3. Graph of platelet surface density on five different films as determined by the LDH assay under flow conditions. Error bars represent means ±SD for n=4.
Figure 7.4. SEMs of platelet surface coverage on a.) glass and b.) silicone rubber under static conditions.
Figure 7.5. SEMs of platelet surface coverage on a.) PPF. b.) 50/10K under static conditions.
Figure 7.6. SEMs of platelet surface coverage on a.) glass under static conditions and b.) glass under flow conditions.
7.4 Discussion

We examined platelet adhesion to P(PF-co-EG) hydrogel films under both static and flow conditions. We compared the platelet adhesion and morphology on these surfaces as well as glass, silicone rubber, and the PPF homopolymer. In addition, we altered the copolymer compositions in order to assess what effects the weight percent PEG as well as the molecular weight of PEG within the copolymer had on the resulting adhesion and morphology. It has been shown previously that increasing either the PEG content or molecular weight above a certain value decreased the adhesion on PEG copolyester hydrogels (Sawhney et al., 1993). It has been suggested that increased hydrophilicity and/or the high mobility of PEG chains in water may be important in decreasing adhesion (Bailey and Koleske, 1976). In order to correlate hydrophilicity to platelet adhesion, we also measured the water contact angles in air of the equilibrium swollen copolymer films. There did not appear to be any differences in hydrophilicity.

All of the copolymer formulations had dramatically fewer platelets adherent compared to PPF homopolymer as determined by the $^{111}$indium oxine-labeled platelet adhesion assay under static conditions. This demonstrates the efficacy of PEG copolymerization in prevention of platelet adhesion. Although, there were some differences in the platelet adhesion among the three copolymers under static conditions, there were no differences in surface contact angles among the copolymer hydrogels; all three formulations were shown to be extremely hydrophilic. As evidenced by the $^{111}$indium oxine assay, however, increasing either the weight percent or the molecular weight of PEG caused a significant decrease in adhesion compared to glass. This suggests that mechanisms other than hydrophilicity have an important effect on platelet adhesion such as steric hindrance or surface free energy. It is important also to note that only two different PEG weight percentages and molecular weights were examined. Although, the weight percentages were chosen based on the limits of the copolymer fabrication technique and the
molecular weights were chosen based on previous work (Suggs et al., submitted), they may not demonstrate the range of effects that PEG can elicit on platelet adhesion.

The LDH assay was compared with the $^{111}$indium oxine assay in order to develop a non-radioactive, reliable assay to measure platelet attachment in the high volume flow studies. The LDH assay has previously been shown to be an effective method for counting adhered platelets, as compared to the radioactively-labeled indium technique (Tamada et al., 1995). In our studies, we show that the LDH assay does not give statistically different values from the $^{111}$indium oxine assay except in the case of PPF. We believe one or both of the following events may be occurring in the case of PPF: 1. The adhered platelets may be activated and release their LDH prior to lysis. 2. The LDH enzyme may become adsorbed and/or inactivated on the polymer surface. In order to examine event 1, we performed the LDH assay on the supernatants of all the films immediately after incubation. There were no statistical differences. (Data not shown.) There is some morphological evidence for platelet disruption at the PPF surface, as shown by debris evident in the scanning electron micrographs. (See Figure 7.5.) This shows that event 1 is probably not the single cause for the discrepancy in the case of PPF.

The flow studies were quantified using the LDH assay on glass, silicone rubber, and the three copolymer formulations. Two important differences prohibit direct correlations to be made between the static and flow studies. First, there are proteins present in whole blood that are not present in the buffered platelet suspension. These proteins, when adsorbed to materials, determine the extent of adhesion and aggregation, and they may affect the test surfaces differently (Andrade et al., 1987). Second, flow itself affects platelet adhesion and the number of platelets may be surface dependent (Ross and McIntire, 1995). Changes in relative total adherent platelets, however, can be demonstrated. The differences between the reference materials and the copolymer films were more pronounced in the studies conducted under flow conditions, and in fact, the number of platelets was not statistically different from zero for all three copolymers. This
suggests that the platelet adhesion to the copolymers in the static case was relatively weak, and that in a more physiologically relevant environment, the platelets may detach readily.

The measurement of platelet attachment does not differentiate between platelets which have adhered and those which have aggregated. Morphology was therefore examined qualitatively using SEM and ESEM. In the static case, glass was the only surface which showed aggregation as evidenced by thrombus formation. Similar aggregates were seen in the flow study; however, the platelets exhibited the more rounded, flattened shape typical of a highly activated platelet (Park et al., 1990). Morphological differences may also serve to explain the relatively large surface coverage of platelets on silicone rubber under flow conditions. Even though the total number of attached platelets relative to glass was equal in the flow studies, no changes were evident in the platelet morphology between static and flow as were seen on glass. This indicates that the platelets were not highly activated, and the silicone rubber was relatively less thrombogenic than glass (Amiji and Park, 1992). The copolymer surfaces showed no evidence of aggregation either in the static or the flow studies. These promising results indicate that the P(PF-co-EG) materials may have successful use in vascular applications where prevention of thrombosis is critical.

7.5 Conclusions

P(PF-co-EG) hydrogel films showed decreased platelet attachment compared to PPF homopolymer under static adhesion conditions. Increasing weight percent PEG or PEG molecular weight caused a significant decrease in attachment as determined by $^{111}$indium oxine-labeled platelets. An enzymatic assay based on lactate dehydrogenase (LDH) was shown to be an effective and reproducible method for counting platelets on the copolymer surfaces. This assay was used to examine platelet adhesion in a flow system using whole blood. The platelets exhibited reduced attachment relative to the reference
materials of glass and silicone rubber under the conditions of the flow study. In addition, platelet morphology was examined for both studies and morphological changes were only apparent on the glass surfaces. The platelets adherent on the copolymer films were rounded and there was no evidence of thrombus formation.
CHAPTER EIGHT

CYTOTOXICITY AND IN VIVO BIOCOMPATIBILITY OF POLY(PROPYLENE FUMARATE-CO-ETHYLENE GLYCOL) HYDROGELS

8.1 Introduction

In the determination of the biocompatibility of an injectable, biodegradable material, there are a number of important considerations. First is the possible contribution of toxic leachable products. These can include: unreacted monomer, byproducts from the radical crosslinking initiation, residual solvents, and potentially unreacted polymer. Second is the composition of the material itself. The relative amounts and segment lengths of the hydrophobic and hydrophilic components can have a significant effect on adhesion as demonstrated in previous studies. Finally, the release of soluble degradation products or degradation accelerated by cellular interaction can affect biocompatibility results. We have attempted to isolate these different effects by evaluating each contribution separately. The cytotoxicity due solely to leachable products was examined in vitro prior to evaluation in an in vivo model. In addition, these studies were performed for time periods at which no degradation products would be produced as shown by previous studies.

This study details the cellular response to P(PF-co-EG) hydrogel films of both endothelial cells in culture and inflammatory cells in an in vivo cage implantation model. The composition and molecular weights of the PEG component were varied in order to assess their effects on the resulting tissue response. The in vitro study was specifically designed to elucidate the effects of toxic leachable products from the copolymer films initially. The cage implantation study allowed for the isolation of effects due to inflammation from leachable products as well as the interaction of adherent cells on the material surface itself. This was evaluated in terms of the humoral and cellular responses,
enzyme release, cell-material interactions including cell adhesion and foreign-body giant cell (FBGC) formation on the surface of the material. The cage implantation system permits the observation of biological changes in the acute and chronic inflammatory cell responses due to the presence of the biomaterial.

8.2 Materials and Methods

8.2.a Copolymer Synthesis

PPF was produced as previously described (Peter et al., submitted). Briefly, fumaryl chloride (Aldrich, Milwaukee, WI) in chloroform solution (Aldrich) was added slowly to a slurry of propylene glycol (Acros, Pittsburgh, PA) and potassium carbonate (Fisher, Pittsburgh, PA) in a double volume of chloroform. The potassium carbonate was in a 2:1 molar excess with propylene glycol, and the ratio of fumaryl chloride to propylene glycol was 1:3. The reaction occurred at room temperature and was vigorously stirred with the use of an mechanical stirrer. The resulting intermediate oligomeric ester was centrifuged in order to remove the potassium carbonate. This intermediate was then placed in a reaction vessel at 160°C under a vacuum of 110 mmHg until the appropriate molecular weight was achieved. The copolymer was then formed through the addition of commercially available PEG (Aldrich) (Suggs et al., 1997b). Purification was performed by solution precipitation from chloroform into petroleum ether (Fisher). The product was then dried by roto-evaporation. PPF of known molecular weight was used in the formation of three different copolymers. The base formulation, 50/5K, had a composition of 50% PEG by weight with a PEG molecular weight of 4,600. The second formulation, 75/5K, had a higher weight percent PEG of 75%, and the third formulation, 50/10K, had a higher molecular weight of PEG of 10,500.
8.2.b Fabrication of Hydrogel Films

The copolymer was mixed with distilled deionized water (ddH₂O) in a 1:1 ratio (wt/vol). In a separate vial, the initiator, benzoyl peroxide (BP) (Aldrich), was mixed with a vinyl monomer, N-vinyl pyrrolidinone (VP) (Aldrich), at a ratio of 1:0.5:1, copolymer: initiator:monomer (wt/vol/vol). The initiator solution was dissolved into the copolymer solution. An accelerator, N,N-dimethyl-p-toluidine (DMT) (Aldrich), was then added in catalytic amounts (approximately 1 μl). The mixture, while still in a fluid state, was poured into Teflon molds which had been machined to a depth of 1 mm and covered with a glass coverslip. The copolymers were then allowed to cure, and the resulting films were removed from the molds and cut to the appropriate dimensions. For the cytotoxicity tests, a cork borer was used to cut disks of 10 mm diameter. The films for the in vivo study were cut to 5 mm x 10 mm. All of the films were allowed to air dry overnight and then stored desiccated prior to use.

8.2.c Cytotoxicity Tests

An endothelial cell line, ECV-304, passages 37-90, was used. (ATCC, Manassas, VA) The cells were stored under liquid nitrogen and cultured as required. They were cultured in indicator-free Dulbecco’s modified eagle medium (DMEM) (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD), Fungizone (Gibco), PSN antibiotic mixture (Gibco), and 25 mM HEPES buffer (Sigma, St. Louis, MO) at 37°C in a 5% CO₂ atmosphere. Once 80% confluency was reached, trypsin (Gibco) was used to harvest the cells, and they were then subcultured onto 24-well plates at a density of 50-60 x 10³ cells/cm² for 24 hr. Cell culture inserts (0.4 mm pore size, Becton-Dickinson, France) were placed onto the tissue culture wells, and UV sterilized copolymer disks were then put into the inserts. The
cultures were incubated for the appropriate time period and then assayed for cell density and proliferation.

8.2.d Cell Density Assay

After incubation with the copolymer disks, the culture media was aspirated and the cells were rinsed three times with phosphate-buffered saline (PBS). The entire culture plate was then frozen at -80°C until analysis was performed. At the time of analysis, the cell samples were thawed and removed from the culture plate using 1.4 ml cold 10 mM EDTA (pH 12.3). The samples were placed into 5 ml RIA tubes and homogenized using a tissue homogenizer. The tubes were incubated at 37°C for 20 min and then chilled to 0°C. The pH was adjusted to 7.0 using 1 M KH₂PO₄. Prior to taking fluorescence readings, 1.5 ml of a Hoechst solution (200 ng/ml) (Polysciences (Warrington, PA) was added. The emission wavelength was read at 455 nm and the excitation wavelength at 350 nm on an Amino-Bowman Series 2 Luminescence Spectrometer (SLM-Aminco, Urbana, IL). Cell and DNA solutions of known concentrations were also run as standards (West et al., 1985).

8.2.e Proliferation Assays

[^H-Thymidine Uptake.][^H-thymidine uptake analysis was performed as previously described (Wu et al., 1996). Immediately after the copolymer disks were placed into the tissue culture plates, 3μl of[^H-thymidine was added to selected wells. At the appropriate time point, the inserts were removed, the media was aspirated, and the wells were rinsed three times with PBS. Trypsin was then added for 10 min at 37°C to detach adherent cells. The wells were diluted with PBS and the resulting solution was
thoroughly mixed and added to a scintillation cocktail. The samples were then counted for 2 min each on a Minaxi β Tricarb 4000 Series liquid scintillation counter (Parkard, Laguna Hills, CA).

**Alamar Blue Assay.** Proliferation was assessed using the colorimetric indicator, Alamar Blue (Alamar BioSciences Inc., Sacramento, CA) (Nikolaychik et al., 1996). Alamar Blue indicator was added to the tissue culture well at a ratio of 200 µl indicator to 2 ml of media. The plates were then incubated for 2 hr at 37°C. The culture inserts were removed, and the media was placed into cuvettes. Absorbances were measured on a Hewlett Packard 8452A Diode Array Spectrophotometer (Germany) at wavelengths of 560 and 600 nm. Controls containing only media and Alamar Blue reagent which had also been incubated for 2 hrs were also measured.

**MTT Assay.** The MTT assay was performed as previously described (Ciapetti et al., 1993). Briefly, a stock solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT, was made with PBS at a concentration of 5mg/ml and sterilized. Aliquots of this solution at a ratio of 100 µl per 1 ml of media were added to select wells. The plates were then incubated for 3 hrs at 37°C. The inserts were removed, and the cells were rinsed three times with PBS. A solution of DMSO and isopropanol was added to lyse the cells and dissolve the dye. The samples were then placed into cuvettes. Absorbances were measured on a Hewlett Packard 8452A Diode Array Spectrophotometer (Germany) at a wavelength of 560 nm.
8.2.f Cage Implant System

The inflammatory response was assessed over 21 days in an \textit{in vivo} model system known as the 'cage implant system,' described previously in detail (Marchant et al., 1983; Ronneberger et al., 1996). Type 304 stainless steel wire mesh (size 24, diameter 0.011 in, Cleveland Wire and Mesh Co., OH) was fabricated into cylindrical cages approximately 3 cm in length and 1 cm in diameter. Dehydrated copolymer films were placed inside the cages, the wire ends directed inward, and the entire device sterilized with ethylene oxide. Empty cages were also prepared as controls. The cages were then implanted into 3 month old, female Sprague-Dawley rats (250-300 g, Charles River Laboratories, MA). The rats were anesthetized with ethyl ether, and two cages were implanted subcutaneously in the back of each rat, at the level of the panniculus carnosus. The rats were given food and water \textit{ad libidum} and allowed free movement within their cages. They were housed in the Animal Research Center at Case Western University. NIH guidelines for the care and use of laboratory animals were observed.

8.2.g Quantitative Exudate Analysis

The inflammatory exudate that accumulated within the cages was aspirated using a 27.5 G needle with a 1 cc syringe (Becton-Dickinson, NJ). Exudate withdrawal was achieved by inserting the needle into the cage cap at the cephalad end of the cage, and allowing the exudate to drain into the syringe. Duplicate implants permitted two withdrawals per rat at non-consecutive time points. The volume of exudate withdrawn did not exceed 0.2 ml.

The exudate was checked for infection on brain-heart infusion plates (University Hospitals, OH). The total cell count was then taken using a hemacytometer. Individual 200 µl aliquots of solution containing exudate and RPMI (Gibco) were spun at 700 rpm
for 10 min onto precleaned glass microslides using a Cytospin 2 cytocentrifuge (Shandon Inc., PA). Each slide was stained with modified Wright's stain (Sigma Diagnostics, MO). The Wright's stain was used to identify and differentially count polymorphonuclear leukocytes (PMNs), monocytes, and lymphocytes present in the exudate. A total of 200 cells were counted for each slide and overall concentrations for each leukocyte type were calculated.

8.2.h Implant Retrieval and Film Analysis

Rats were sacrificed at 7 and 21 days postimplantation by ether overdose. The cages were retrieved, cut open, and the films were carefully removed. The films were then placed in 5 ml of fixative containing 0.1 M cacodylate buffer with 2.5% gluteraldehyde (Sigma Chemical Co., MO) and 4% sucrose, and stored at 4°C. After 12 hrs, the copolymer specimens were bisected using a razor blade. One half of each of the films was placed in phosphate-buffered saline (PBS) at 4°C to be used for light microscopy. The other half of each of the films was rinsed in distilled deionized water, then serially dehydrated at 5 min intervals from 30% to 100% ethanol. Afterwards, the samples were soaked in hexamethyldisilazane (HMDS) (Sigma Chemical Co.) for 2-15 min increments. The samples were vacuum-dried for 48 hrs and sputter-coated with a 200 Å layer of Au-Pd using a IB3 Ion Coater (RMC-Eiko). Low voltage SEM evaluations were made using a JEOL 840A (JEOL, Japan) scanning electron microscope.

The remaining specimens were removed from the buffer solution and stained with modified Wright's stain. The specimens were then immersed in ddH₂O for 24 hrs in order to remove excess stain from the films themselves. The density of adherent cells (cell no./sample area) on each retrieved specimen was determined using Sigma Scan Pro software (SPSS, IL) coupled to an Olympus BH-2 light microscope (Olympus, Japan).
Quantitative measurements were obtained from 10 representative areas per sample (5 per side) avoiding measurements at the film edges or material defects.

8.3 Results

8.3.a Cytotoxicity Tests

Endothelial cell densities were determined at 1, 4, and 7 days after exposure to copolymer films as shown in Figure 8.1. The results from this assay show a greater number of cells present in the control wells. The controls show an increase in cell number until day 4 followed by a leveling off due to contact inhibition. All of the copolymer films showed similar density values.

Results from the proliferation assays are shown in Figures 8.2, 8.3, and 8.4. The results from the $^{3}$H-thymidine incorporation show significantly higher values for the controls with no material present, relative to the cells exposed to the copolymer. The controls show a typical pattern where proliferation increases until 4 days, where it begins to plateau. At day 1, the cells exposed to copolymer 50/5K show little activity with copolymers 75/5K and 50/10K slightly higher. At day 4, the values for the test wells are very low, but begin to increase by day 7.
Figure 8.1. Change in cell density over 7 days after exposure to three different copolymer formulations. Error bars represent mean±SD for n=3.
Figure 8.2. Change in cellular proliferation as determined by $^3$H-thymidine incorporation over 7 days after exposure to three different copolymer formulations. Error bars represent means±SD for n=3.
Figure 8.3. Change in cellular proliferation as determined by Alamar Blue reduction over 7 days after exposure to three different copolymer formulations. Error bars represent means±SD for n=3.
Figure 8.4. Change in cellular proliferation as determined by MTT absorbance over 7 days after exposure to three different copolymer formulations. Error bars represent means±SD for n=3.
Similar results are obtained using the Alamar Blue indicator. Day 4 shows no cellular proliferation under copolymer 50/5K with somewhat greater values for cells exposed to copolymers 75/5K and 50/10K. Unlike the \(^3\)H-thymidine assay, the Alamar Blue assay shows a gradual increase in cellular activity throughout the time course. This discrepancy may be due to the fact that \(^3\)H-thymidine incorporation was measured over 24 hrs while Alamar Blue reduction was measured over 2 hrs. The initial cytotoxicity over the first 24 hrs may be gradual as the leachable components in the copolymer were solubilized, resulting in inflated activity values for \(^3\)H-thymidine incorporation for day 1. In addition, the control values for the Alamar Blue assay did not exhibit a pattern characteristic of proliferation, but gradually decreased over the 7 day time course.

The MTT assay was found to be the least typical proliferation assay, showing no plateau during the course of the study. The MTT test, however, was found to be sensitive only to cell concentrations of approximately \(1.2 \times 10^5\) cells/ml or more. Controls with no copolymers present showed a constant increase in absorbance from day 1 to day 7. Test wells showed very little proliferation at 1 day and 4 days. At 7 days, however, there was some evidence of proliferation, although only statistically different from zero for copolymer 50/10K.


8.3.b Exudate Analysis

After withdrawal of the exudate from the cage implants at days 4, 7, 14, and 21, the differential leukocyte counts were determined for each material. The results from these counts are shown in Table 8.1. For the empty cage controls, the total leukocyte concentration (TLC) in the exudate decreased as the implantation time increased, indicating a mild inflammatory reaction to the cage proceeding towards resolution. (Figure 8.5) This trend was also observed for all materials. There were no statistical differences found in the exudate of the polymer implants than the empty cage controls at any time point. At 21
days, the leukocyte concentration of the empty cage controls showed a slight increase. This is also true for copolymer 50/5K, but not for the other two copolymers.

Even though all of the copolymers show greater PMN counts that the control, the only statistical difference was between copolymer 50/10K and the control at 4 days. The decrease in total leukocyte count over the time course is due primarily due to macrophage disappearance. The slight increase in leukocyte count for the controls and copolymer 50/5K at 21 days is due to contributions from both lymphocytes and the renewed presence of macrophages. The lymphocyte count remained relatively low throughout the time course. There were, however, no statistically significant differences in lymphocyte or macrophage concentrations except for copolymer 75/5K and the control at 4 days.

The alkaline phosphate activity over the 21 day time course is shown graphically in Figure 8.6. The activity values show a gradual decrease for all implants which was probably caused by the reduced concentration of PMNs. There were no statistically significant differences between any of the copolymers and the controls for any time point. Acid phosphatase activity is a measure of the action of macrophages. The graph of acid phosphatase activity shown in Figure 8.7 again correlates to the macrophage concentration as determined from differential leukocyte counts. A gradual decrease occurred for all implants up to 14 days. At 21 days, there was a slight increase for all implants except copolymer 75/5K, again with no significant differences between polymers and controls.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Implantation Time (days)</th>
<th>TLC (cells/μl)</th>
<th>PMN (cells/μl)</th>
<th>Macrophage (cells/μl)</th>
<th>Lymphocyte (cells/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/5K</td>
<td>4</td>
<td>73.3 ± 34.0</td>
<td>10.5 ± 3.8</td>
<td>60.2 ± 29.2</td>
<td>2.7 ± 2.0</td>
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<tr>
<td></td>
<td>7</td>
<td>11.7 ± 2.9</td>
<td>1.8 ± 0.8</td>
<td>7.8 ± 1.8</td>
<td>2.1 ± 0.7</td>
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<tr>
<td></td>
<td>14</td>
<td>6.7 ± 2.9</td>
<td>0.2 ± 0.2</td>
<td>0.9 ± 0.7</td>
<td>4.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>13.3 ± 7.6</td>
<td>0.1 ± 0.1</td>
<td>2.6 ± 1.8</td>
<td>10.6 ± 5.8</td>
</tr>
<tr>
<td>75/5K</td>
<td>4</td>
<td>33.3 ± 11.5</td>
<td>6.2 ± 7.0</td>
<td>25.6 ± 8.6*</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td></td>
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<td>2.1 ± 2.7</td>
<td>6.6 ± 5.0</td>
<td>1.3 ± 1.1</td>
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<td>0.3 ± 0.5</td>
<td>1.0 ± 1.2</td>
<td>8.7 ± 11.5</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>6.7 ± 2.9</td>
<td>0.1 ± 0.0</td>
<td>0.8 ± 0.4</td>
<td>5.8 ± 2.5</td>
</tr>
<tr>
<td>50/10K</td>
<td>4</td>
<td>60.0 ± 13.2</td>
<td>8.8 ± 0.9*</td>
<td>48.9 ± 12.6</td>
<td>2.1 ± 0.7</td>
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<tr>
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<td>10.0 ± 5.0</td>
<td>0.8 ± 0.5</td>
<td>8.0 ± 4.3</td>
<td>1.1 ± 0.5</td>
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<tr>
<td></td>
<td>14</td>
<td>8.3 ± 10.4</td>
<td>1.1 ± 1.6</td>
<td>1.4 ± 1.8</td>
<td>5.8 ± 6.9</td>
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<tr>
<td></td>
<td>21</td>
<td>3.3 ± 3.0</td>
<td>0.1 ± 0.1</td>
<td>0.3 ± 0.3</td>
<td>3.0 ± 2.6</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>55.0 ± 8.7</td>
<td>4.6 ± 0.8</td>
<td>48.6 ± 9.3</td>
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<td>14</td>
<td>5.0 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
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<td>21</td>
<td>20.0 ± 15.0</td>
<td>0.5 ± 0.5</td>
<td>2.1 ± 2.4</td>
<td>17.3 ± 12.4</td>
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</table>

Table 8.1. Differential leukocyte counts at different time points following implantation of copolymer films. (Values are mean ± standard deviation.) (* denotes statistically significant differences from controls.)
Figure 8.5. Total leukocyte count (TLC) over 21 days for three different copolymer formulations after cage implantation. (See Table 8.1) Error bars represent means±SD for n=3.
Figure 8.6. Exudate alkaline phosphatase over 21 days for three different copolymer formulations after cage implantation. Error bars represent means±SD for n=3.
Figure 8.7. Exudate acid phosphatase over 21 days for three different copolymer formulations after cage implantation. Error bars represent means±SD for n=3.
8.3.c Film Surface Analysis

Table 8.2 shows the results from the film surface analysis. Cells on the material surfaces were analyzed at 7 and 21 days after implantation. The film coverage was not evenly distributed and was concentrated primarily at the edges of the films and in film defects. (Figure 8.8) Cell density was highest for copolymers 75/5K and 50/10K, while average cell size was relatively consistent for all copolymers at both time points. There were no cells larger than 0.003 mm², which has previously been used as minimum value for the quantification of giant cells (Marchant et al., 1984). The percentage of film covered by macrophages was also quantified. The highest coverage attained at 21 days was 4.5% by copolymer 50/10K. There were no statistically significant differences from 7 to 21 days in cells density for any of the copolymers. The average cell area was the same for copolymers 50/5K and 75/5K, but not 50/10K from 7 to 21 days. Scanning electron micrographs are shown in Figure 8.9 and demonstrate singlet macrophages with evidence of some spreading but no fusion to form giant cells. No multinuclear cells were found on the copolymer film surfaces.

8.4 Discussion

Three block copolymer formulations of P(PF-co-EG) were assessed in terms of in vitro cytotoxicity and in vivo biocompatibility. The composition and molecular weight of the PEG component was varied in an effort to quantify their effects on leachable compounds on cultured endothelial cells. These factors were also examined in a rat cage implant model. The tissue reaction of the different copolymers was shown by exudate analysis and film surface analysis and compared to empty cage controls.
<table>
<thead>
<tr>
<th>Polymer</th>
<th>Implantation Time (days)</th>
<th>Cell Density (cells/cm²)</th>
<th>Cell Size (mm² x 10⁴)</th>
<th>Film Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/5K</td>
<td>7</td>
<td>14900 ± 4400</td>
<td>2.4 ± 1.2</td>
<td>3.3 ± 1.2</td>
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<tr>
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<td>21</td>
<td>15300 ± 8400</td>
<td>1.7 ± 0.8</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>75/5K</td>
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<td>35400 ± 25500</td>
<td>2.0 ± 0.9</td>
<td>7.8 ± 7.3</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>16600 ± 15000</td>
<td>1.9 ± 0.7</td>
<td>3.7 ± 4.0</td>
</tr>
<tr>
<td>50/10K</td>
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<td>28800 ± 11600</td>
<td>1.4 ± 0.2</td>
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<tr>
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<td>16400 ± 2700</td>
<td>2.6 ± 0.7</td>
<td>4.5 ± 1.5</td>
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</table>

Table 8.2. Film surface coverage at two different time points following implantation of copolymer films. (Values are mean ± standard deviation.)
Figure 8.8. Film surface coverage by macrophages over 21 days for three different copolymer formulations after cage implantation. (See Table 8.2) Error bars represent means±SD for n=3.
Figure 8.9. Representative scanning electron micrographs (SEMs) of macrophages adherent on copolymer 75/5K showing singlet cells without any evidence of giant cell formation.
The cell density and proliferation data shows significant toxicity due to the presence of copolymer films. All of the assays performed demonstrated low cell numbers for the test wells at 1 and 4 days. At 7 days, however, all of the data showed significant increases. This is consistent with toxic leachable compounds being solubilized early, with the cell population recovering after 1 week. No significant differences due to copolymer composition was noted at the later time points, however, copolymer 50/5K showed the highest level of toxicity at 1 day for all of the assays performed. This composition has the highest percentage of the PPF component. Possible causes for this response may be increased levels of unreacted monomer due to the higher numbers of fumarate double bonds available for crosslinking. Also, more crosslinks may result in additional byproducts from the radical initiation such as benzoic acid.

The P(PF-co-EG) hydrogel films did not cause any significant variation in the inflammatory reaction elicited by the cage implantation as characterized by leukocytes in the exudate. Others have shown that materials with toxic leachable products can cause increased leukocyte concentrations in the cage implant (Marchant et al., 1986). Apparent differences between in vitro and in vivo studies may be due solely to the high localized concentration of cytotoxic products in culture versus a more diluted case in the cage implant. For both studies, the copolymer with the highest PPF content, 50/5K, showed the most dramatic response. This material proved to be the most cytotoxic according to cell density and proliferation. Also, 50/5K had the highest number of leukocytes present and alkaline phosphatase activity at 4 days. In addition, copolymer 50/5K showed an increase in leukocyte concentration at 21 days. This indicates that increasing PEG molecular weight or content can decrease acute inflammation.

PMN concentration is high during the initial phases of inflammation. Higher PMN counts are indicative of an enhanced acute inflammatory response, but in this case may be the result of surgical procedure creating a more severe injury. Macrophages then accumulate in the exudate and as shown at day 4, are the dominant cell type for all
implants. Alkaline phosphatase is located with in PMN granules and is an indicator of lysis or activation. This enzyme is indicative of PMN activity and its decline mirrors that of the PMN concentration and is typical of the resolution of the acute inflammatory phase. Acid phosphatase is located within azurophilic granules of both PMNs and macrophages. The activation of macrophages is characterized by an increase in this enzyme in the exudate. As the concentration of macrophages decreases, so does the acid phosphatase activity and consequently the activity of the macrophages. At 21 days, however, there is a slight resurgence of macrophage activity which may result from increased cell/material interactions at later time points.

The absence of FBGCs on the surface of the copolymer films indicate that these materials are very non-adhesive to cells and do not cause the activation of macrophages. There may, however, be some indication that the materials with higher PEG content or molecular weight are more susceptible to biodegradation. Previous studies showed evidence of particulate formation in the fibrous capsule surrounding copolymer implants in a subcutaneous rat model (Suggs et al., submitted). These fragments were more pronounced in the case of higher molecular weight PEG or higher weight percent PEG copolymers. In this study, although there was no evidence of degradation, copolymers with higher content or molecular weight PEG, 75/5K and 50/10K, showed the highest film surface coverage. In addition, copolymer 75/5K showed significant increases in cell area. Previous data has shown that the number of cells on a biomaterial surface typically decreases while the average area increases due to the fusion of macrophages to form FBGCs. Even though no giant cell formation was observed, differences in surface coverage among the copolymer formulations from 7 to 21 days shows some effects due to the PEG component.

We found that a combination of in vitro and in vivo techniques is necessary in isolating the nature of biomaterial/tissue interactions. There are also many factors which contribute to the biocompatibility of an implantable material. The surface characteristics
and wettability of the polymer contribute to cellular adhesion and FBGC formation, as shown by the variation due to changes in copolymer composition. The presence of leachable products in an injectable system can contribute significantly to the acute inflammatory response, and as the material degrades, degradation products as well as the mode of degradation can effect the foreign-body response.

8.5 Conclusions

P(PF-co-EG) hydrogel films showed significant cytotoxicity towards cultured endothelial cells as evidenced by cell density and proliferation over a 7 day time course. In a 21 day cage implantation model, three copolymer formulations were examined all of which demonstrated excellent biocompatibility both with respect to exudate leukocyte concentration and enzyme analysis. There were slight differences due to copolymer composition. The results showed decreased cytotoxicity and acute inflammation due to increases in either the molecular weight or weight percent of the PEG component. At later time points, even though there was no evidence of degradation, these same copolymers exhibited slightly more surface coverage by macrophages presumably due to increases in cell/biomaterial interactions.
CHAPTER NINE

DEVELOPMENT OF POLY(PROPYLENE FUMARATE-CO-ETHYLENE GLYCOL) AS AN INJECTABLE CARRIER FOR ENDOTHELIAL CELLS

9.1 Introduction

This chapter focuses on the evaluation and development of P(PF-co-EG) copolymers as injectable, \textit{in situ} polymerizable carriers for the delivery of cultured endothelial cells. An \textit{in situ} polymerizable system could potentially be used as a carrier for endothelial cells without the need to preseed cells onto the stent surface. In an ideal case, a mixture of polymer and cell suspension could be injected into the arterial space using a catheter delivery system as shown in Figure 9.1. The material would cure, forming a hydrogel which would allow for the diffusion of nutrients to the cells embedded in the matrix. As the material degraded, the endothelial cells would repopulate the vessel lumen preventing thrombosis and the hyperplastic response characteristic of restenosis.

We examined the feasibility of the copolymer to be injection polymerized \textit{in vivo} in a subcutaneous rat model. The tissue surrounding the copolymer implants was evaluated histologically to determine if the injected copolymers were biocompatible. In addition, endothelial cells were embedded in the copolymer during the crosslinking and assayed for DNA synthesis, a measure of proliferation, over the first 24 hrs. Temperature profiles were also taken during crosslinking for three different copolymer formulations to determine the maximum curing temperature.
Figure 9.1. Diagram of the proposed system for injection polymerization of P(PF-co-EG) hydrogels to the arterial lumen.
9.2 Materials and Methods

9.2.a Copolymer Synthesis

PPF was produced as previously described (Peter et al., submitted). The copolymer was then formed in a transesterification reaction with commercially available PEG (Acros, PA) of varying molecular weights. The reaction was run at 160°C under a nitrogen atmosphere at a pressure of 110 mmHg. The copolymer was purified by solution-precipitation from chloroform to petroleum ether. It was then dried by roto-evaporation. For the temperature profile experiments, three copolymer formulations were fabricated from one molecular weight of PPF. The first was a base formulation with 50% by weight PEG which had a weight average molecular weight of 4,600. The second had a higher weight percent PEG at 75%, and the third had a higher molecular weight PEG at 10,500. These formulations were denoted 50/5K, 75/5K, and 50/10K, respectively. For the injection and embedding studies, copolymer 75/5K was the only formulation used.

9.2.b Temperature Profiles

The temperature of the crosslinking reaction was monitored every fifteen seconds for five minutes (Peter et al., submitted). This was recorded with the use of a thermocouple (Omega, KMTSS-032U-6, Stamford, CT) inserted into a Teflon mold. The mold had inner dimensions 10 mm in diameter and 15 mm in height. The temperature was maintained at 37°C by immersing the mold in a water bath. For each run the copolymer was first mixed with distilled deionized water (ddH₂O) in a 1:1 ratio (wt/vol). The initiator, benzoyl peroxide (BP) (Aldrich), was mixed with a vinyl monomer, N-vinyl pyrrolidinone (VP) (Aldrich), at a 1:0.5:1 ratio of copolymer to initiator to monomer (wt/vol/vol). Both solutions were brought to 37°C by immersion in the water bath. The initiator solution was
mixed with the copolymer solution, and 1μL of N,N-dimethyl-p-toluidine (DMT) (Aldrich) was added. The resulting solution was stirred vigorously and poured into the Teflon mold. Temperatures were recorded from the center of the mold beginning 15 seconds after the addition of DMT.

9.2.c In Vivo Injection Polymerization

Copolymer 75/5K was crosslinked in situ on the backs of male Sprague-Dawley rats (300-350g). They were anesthetized intramuscularly with a cocktail consisting of 3.6mg/ml xylazine, 63.6mg/ml ketamine, 0.07mg/ml atropine at a concentration of 0.23ml/100 g body weight. An amount of 10mg of gentamycin was given at the time of surgery. The animals were prepared by shaving and applying a betadine solution. The copolymer, ddH₂O, benzoyl peroxide, and N-vinyl pyrrolidinone were sterilized by UV irradiation prior to use and combined as described above. The resulting fluid was poured into a 5 cc syringe with an 18 G needle. DMT was then added and the mixture was stirred in the syringe. The syringe plunger was inserted, and approximately 2 ml of copolymer was injected subcutaneously to form a hard, round nodule. Animals were given food and water ad libidum and allowed free movement within their cages. Animals were euthanized with ethyl ether, and the samples were harvested at 3 and 9 weeks. After the animals were euthanized, the tissues surrounding the copolymer films were removed with the copolymers intact whenever possible. These samples were fixed in 10% formalin for at least 48 hours. They were then serially dehydrated and paraffin-embedded prior to sectioning and staining with hematoxylin and eosin (H&E). Animal care protocols followed the NIH guidelines for the care and use of laboratory animals.
9.2d Endothelial Cell Embedding

Endothelial cells, ECV-304, were obtained from ATCC (Mountain Lakes, NJ). They were stored under liquid nitrogen and cultured as required. The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD), Fungizone (GIBCO), PSN antibiotic mixture (Gibco), and 25 mM HEPES buffer (Sigma, St. Louis, MO) at 37°C in a 5% CO₂ atmosphere. Once 80% confluency was reached, trypsin (Gibco) was used to harvest the cells, and they were suspended in media at a concentration of approximately $3 \times 10^6$ cells/ml. The copolymer, ddH₂O, benzoyl peroxide, and N-vinyl pyrrolidinone were sterilized by UV irradiation prior to use and combined as described above. A 200 μl volume of cell suspension was added to some of the copolymer mixtures, while 200 μl of media was added to others as controls. DMT was added with stirring. The resulting fluid was then aliquoted into 24-well tissue culture plates at 0.5 ml per well. Additional media was added to the wells, and the plate was incubated at 37°C for 24 hr.

$^3$H-thymidine uptake analysis was performed as previously described (Wu et al., 1996). Immediately after the copolymers were crosslinked in the tissue culture plates, 3μl of $^3$H-thymidine was added to each well. At 24 hrs, the media was aspirated, and the wells were rinsed three times with PBS. The copolymer disks were then removed from the bottom of the well and added directly to a scintillation cocktail. The samples were then counted for 2 min each on a Minaxi β Tricarb 4000 Series liquid scintillation counter (Parkard, Laguna Hills, CA).

Selected samples were fixed in 10% formalin for at least 48 hours. The entire samples were then stained with hematoxylin and eosin (H&E). They were then
embedded frozen and sectioned to 6 μm using a cryostat. The sections were mounted using wet mounting media and images were taken from slides.

9.2.e Statistical Analysis

Statistical differences between sample populations were evaluated using a student's unpaired t-test with a 95% confidence interval (p<0.05).

9.3 Results

9.3.a Temperature Profiles

Representative temperature profiles of copolymers 50/5K, 75/5K, and 50/10K are shown in Figure 9.2. The average maximum temperature reached was 38.3°C±0.2 for 50/5K, 38.1°C ±0.1 for 75/5K, and 38.1°C ±0.1 for 50/10K. There were, however, no statistical differences among any of the copolymers with respect to the maximum temperature reached. Copolymer formulation 75/5K reached its maximum temperature the fastest in an average of 2.7±0.2 min. Copolymer 50/5K reached its maximum in 3.0±0.4 min, and 50/10K in 3.3±0.3 min. The only statistical difference in the time at which the maximum temperature was achieved was between copolymers 75/5K and 50/10K.
Figure 9.2. Temperature profiles for the crosslinking reaction of three different copolymer formulations.
9.3.b *In Vivo* Injection Polymerization

The rats that received the copolymer injections recovered well from the anesthesia and appeared normal with no signs of systemic shock. The copolymer nodule was firm to the touch throughout the time course. Upon explantation as shown in Figure 9.3, it was evident that the nodule was encased in a fibrous capsule. There was no evidence of redness, swelling, or any fluid exudate. The fibrous capsule was then cut open and the nodule was removed intact with no apparent fragmentation. Upon histological evaluation at three weeks, as shown in Figure 9.4, the tissue displayed minimal chronic response at the polymer/tissue interface. The fibrous capsule was compacted and consisted primarily of fibroblasts and collagen fibers. At nine weeks, the specimens displayed a mature fibrous capsule with a limited foreign body reaction at the implant interface. There was evidence of biodegradation at the polymer surface with macrophages and a few giant cells present.

9.3.c Endothelial Cell Embedding

The incorporation of $^3$H-thymidine after 24 hrs is shown graphically in Figure 9.5. The copolymer itself non-specifically absorbs $^3$H-thymidine which is why the controls consisting of copolymer crosslinked without cells show a certain amount of activity. The copolymers that were crosslinked with cells, however, had a statistically significant increase in the amount of $^3$H-thymidine incorporated. This shows that the endothelial cells survived the crosslinking process and were synthesizing DNA immediately after embedding.
Figure 9.3. Surgical explantation at 9 weeks of a copolymer nodule which had been polymerized \textit{in situ}. 
Figure 9.4. Histological section (H&E) of tissue surrounding the injection polymerized copolymer at 3 weeks.
Figure 9.5. Endothelial cell proliferation 24 hrs after embedding in P(PF-co-EG) hydrogel.
Figure 9.6 shows the presence of endothelial cell nuclei (stained purple by hematoxylin) embedded in the copolymer matrix. The cells occurred singly and were spread out evenly throughout the matrix. The nuclei showed evidence of chromatin organization characteristic of living cells. The cell cytoplasm is not evident and should be stained pink from the eosin stain. This did not occur probably because the eosin is non-specifically absorbed in the cytoplasm. The eosin is also water soluble and may desorb during the rinsing process. The copolymer, however, can not be dehydrated or the structure will be lost.

9.4 Discussion

In the development of an injectable polymer system, an important consideration along with the inherent cytotoxicity of the material itself, is the maximum temperature achieved during the crosslinking or curing process. If the temperature gets too high, there can be subsequent tissue necrosis around the site of the implant. The maximum curing temperature for all three copolymer formulations used in this study did not rise above 38.3°C±0.2 starting from a value of 37°C. In comparison, the maximum curing temperature measured for PMMA bone cement is 94°C (Jefferiss et al., 1975). This indicates that the curing process will not damage surrounding tissues. Also, the tolerance of cultured cells for perturbations in temperatures is an additional restriction in the development of an injectable carrier.
Figure 9.6. Histological section (H&E) of hydrogel matrix showing the nuclei of embedded endothelial cells.
The shape of the temperature profile for copolymer formulation 75/5K appears slightly different than the other two formulations with higher PPF concentrations, having a more gradual increase versus a sharp peak. In addition, the time at which the maximum temperature was reached for copolymer 75/5K was significantly earlier. This is most probably due to the fact that even though 75/5K has fewer sites for crosslinking, the PEG chains may impart more mobility to the copolymer in an aqueous environment and allow the double bonds to come into contact more easily. The greater PEG content may also act as a thermal sink, allowing for the dissipation of heat during the curing process.

The in vivo injection polymerization experiments indicated the ability of the copolymer to be crosslinked in situ. The test subjects did not show any noticeable adverse reaction to the implantation. Histologically, the copolymer implants showed good biocompatibility. There was an infiltration of neutrophils which is characteristic of an acute inflammatory response. A fibrous capsule formed and its development and maturation was evident at 9 weeks. There appeared to be copolymer particles embedded in the fibrous capsule which caused a layer of granular tissue. This layer was active and was limited to the polymer/tissue interface, consisting of foamy macrophages and a few giant cells. The tissue response was similar to that described in Chapter 6 regarding the in vivo degradation of prefabricated copolymer films. In this case, however, there appeared to be a more intense infiltration of neutrophils during the acute stage as well as a thicker fibrous evident at 9 weeks. This is most probably due to any uncrosslinked leachable products which were leached out during the in situ curing. Also, in this case, there is minimal damage to the tissue because of the surgery, and the tissue response can be attributed almost entirely to the presence of the copolymer itself.

There are a number of criteria that need to be addressed in the development of an injectable, in situ polymerizable carrier for cultured cells. The cells must be able to survive the initial crosslinking process. This can include factors such as temperature increases during curing and cytotoxicity from residual solvents or byproducts of radical initiation.
The cells must also be able to survive and proliferate within the polymer matrix. Issues at this stage include: cytotoxic degradation products, localized decreases in pH, and most importantly, diffusional limitations. In this study, we addressed the ability of endothelial cells to survive the initial crosslinking process, specifically, 24 hrs after incorporation. There was cellular proliferation during this time as shown by ³H-thymidine incorporation. In addition the nuclear morphology was characteristic of living cells. However, similar studies for longer time points showed no proliferation. (Data not shown.) This indicates that while endothelial cells can survive the initial phase, some component of the reaction is destructive. Over the short time course investigated, cell death is most probably not caused by degradation products or diffusional limitations. In addition, the low maximum crosslinking temperatures reached indicate that the contribution of heat release is not an important. Based on these and previous studies, we feel that cytotoxic leachable products such as: residual solvents, unreacted monomer, or initiation byproducts cause cellular necrosis within the copolymer matrix.

9.5 Conclusions

Based on initial evaluation, P(PF-co-EG) shows potential as an injectable carrier for cultured endothelial cells in cardiovascular applications. The copolymer demonstrated the ability to be crosslinked in vivo. Over a nine week time course, the injection polymerized P(PF-co-EG) nodules exhibited a normal inflammatory and wound healing response in a subcutaneous rat model. There was fibrous encapsulation along with evidence of particulate formation within the capsule. Endothelial cells were embedded in the copolymer matrix and showed proliferation as evidenced by ³H-thymidine incorporation over 24 hrs. The cell nuclei were visualized within the matrix with hematoxylin staining and demonstrated chromatin organization typical of living cells. Subsequent cell death was most probably not caused by thermal increases as shown by temperature profiles taken on
three different copolymer formulations in which the maximum temperature did not rise about 38.3°C. This temperature did not depend on the amount or molecular weight of PEG, but the shape of the profile did show significant changes caused by the amount of PEG.
CHAPTER TEN

CONCLUSIONS AND FUTURE WORK

P(PF-co-EG) was developed for cardiovascular applications. Specifically, this material was designed to function as an injectable, biodegradable stent to prevent restenosis and repave the arterial lumen following PTCA. The PPF was chosen to serve as the crosslinkable, degradable component, while the PEG served to impart flexibility, biocompatibility, and nonthrombogenic characteristics. The material was characterized in its linear form to verify connectivity as well as thermal and solubility properties. The copolymer was then crosslinked which resulted in the formation of a hydrogel. The hydrogel matrix was characterized in terms of swelling behavior, mechanical properties, and degradation characteristics. These hydrogels were shown to be highly crosslinked with moderate swelling and relatively good mechanical properties relative to other hydrogels. The PEG copolyester degraded in bulk and maintained significant mechanical properties during degradation.

The cellular and tissue response of P(PF-co-EG) hydrogels was evaluated in a number of different in vitro and in vivo models. The adhesion of platelets served as a predictor of thrombogenicity, and the presence of the PEG component was shown to impart nonthrombogenic characteristics to the resulting copolymer as shown both in static and under flow conditions. As an injectable material, it was important to examine toxic effects of any residual products after crosslinking. The copolymer was shown to be somewhat cytotoxic in vitro, however, in an in vivo model there was no evidence that inflammation was increased due to toxic byproducts. The adhesion and activation of leukocytes was quantified as a measure of the biocompatibility of these materials. They showed excellent biocompatibility with minimal foreign body reaction. In addition, in vivo and in vitro models were used to verify the injectability of the P(PF-co-EG).
Throughout the course of these studies, an effort was made to optimize this material for its intended use. The components of the crosslinking reaction including the monomer, the initiator, the presence or absence of an accelerator, and the presence or absence of water have been varied extensively. The composition of the linear chain copolymer has been varied in terms of molecular weight, reaction times, and weight percent of the two homopolymers. In addition, the PPF reaction itself has been improved to remove any side products and provide additional sites for crosslinking. It is important to pursue modifications in the copolymer composition for a number of reasons. First, the block copolymer structure provides an opportunity to vary the mechanical, degradative, and biological properties of the resulting hydrogel to fulfill a wide range of functions. Secondly, the crosslinking monomer used is known to be toxic and is the probable contributor to the cytotoxicity of the unleached copolymer hydrogel. It would be advantageous to remove it or modify the crosslinking reaction. Lastly, the uniqueness of this material is in the fact that there are crosslinkable groups all along the copolymer backbone. Theoretically, an optimum composition can be reached which provides the best mechanical properties while preventing adhesion and allowing hydration and diffusion through the matrix.

Another unique feature of P(PF-co-EG) is its low crosslinking temperature. This feature lends itself to the embedding of bioactive molecules or cells during the crosslinking process. In our initial studies, we showed that endothelial cells could be incorporated into the matrix and proliferate. This result has potential for a number of tissue engineering applications. Endothelial cells could be injection polymerized along with the copolymer and as the stent formed in the lumen, these cells would be dispersed throughout the matrix. As the material degraded, the endothelial cells could repopulate the damaged vessel and serve to repave the lumen with an intact layer of endothelial cells. The copolymer could also be used to deliver genetically altered cells or genetic material for gene therapy applications. The advantage of this type of therapy is the easy of delivery and
noninvasiveness of the implantation. P(PF-co-EG) hydrogels could also be used as drug delivery vehicles. Again, the injectability of these materials could be used to localize drugs to specific target areas such as tumors or other tubular tissues. Finally, an exciting application for this copolymer could be in cartilage, adipose, or other tissue which fulfills a structural but not load-bearing function. Autologous cells could be incorporated into the copolymer matrix and form \textit{in situ} providing a scaffold for the repopulation of native tissue. We believe that this type of non-surgical tissue regeneration represents another generation of tissue engineering.


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VITAE

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Laura has authored seven peer-reviewed publications, two book chapters, and two patents. She has presented her work at seven technical conferences and has supervised five undergraduate research assistants. She was the recipient of a NASA NSCORT training grant as well as a NIH training grant during her graduate studies. She received the Hershel M. Rich award for outstanding patent at Rice University for the patent, “Poly(Propylene Fumarate-co-Ethylene Oxide),” as well as the Southern Biomedical Engineering Conference award for best paper by a PhD candidate. She has lectured for several courses at Rice University and looks forward to a career in academia.