RICE UNIVERSITY

SYNTHESIS AND CHARACTERIZATION OF AN INJECTABLE COPOLYMER HYDROGEL FOR CARDIOVASCULAR APPLICATIONS

by

Albert K. Shung

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

MASTERS OF SCIENCE

APPROVED, THESIS COMMITTEE:

Antonios G. Mikos, Ph.D. (Advisor)
Professor
Department of Bioengineering
Rice University

Jennifer L. West, Ph.D.
Associate Professor
Department of Bioengineering
Rice University

Kyriacos Zygourakis, Ph.D.
Professor
Department of Chemical Engineering
Rice University

Bahman Anvari, Ph.D.
Assistant Professor
Department of Bioengineering
Rice University

Houston, Texas
June, 2002
Abstract

SYNTHESIS AND CHARACTERIZATION OF AN INJECTABLE COPOLYMER HYDROGEL FOR CARDIOVASCULAR APPLICATIONS

by

Albert K. Shung

In this thesis, a novel injectable copolymer hydrogel was developed that may be suitable for cardiovascular applications. This material consists of a triblock copolymer of poly(propylene fumarate) and poly(ethylene glycol). The first part of this work involved characterizing the kinetics of the poly(propylene fumarate) (PPF) reaction using zinc chloride as a catalyst. The reaction kinetics were shown to be dependent on the reaction temperature. The second part of the work involved characterizing various properties of the hydrogel by varying components of the water soluble crosslinking system and the copolymer structure. The properties examined included equilibrium water content, sol fraction, mechanical strength, onset of gelation, molecular weight between crosslinks, and endothelial cell adhesion. In addition, these hydrogels were modified with bioactive peptides to test for their feasibility as a future promoter of endothelial cell adhesion and migration. The results from all these studies show that this novel injectable hydrogel may be suitable for cardiovascular applications and other tissue engineering applications.
Acknowledgements

"Two are better than one, because they have a good return for their work: If one falls down, his friend can help him up. But pity the man who falls and has no one to help him up!" Ecclesiastes 4:9-10

In my 5 years at Rice University, I have interacted with many different people who have come and gone through the Mikos lab. I am not exaggerating when I say that each one of you has made an impact on my life in one way or the other. Listed below are just a few of the people that have been especially significant to me. Believe me when I say that words cannot do justice to how I truly feel.

To my beautiful wife, Rini, who was an angel sent to me by God. Thank you for all of your unconditional support and love in all that I have done and will do. I would not be the man that I am today if it weren't for you. I love you and always will.

To my good friend and helper, Dr. Seongbong "Sean" Jo. I can honestly say that all the practical organic chemistry and synthesis I know I owe to the expertise of this good and generous man. Thank you for always being there to listen and give advice. God bless your future endeavors!

To my classmate and fellow downstairs cohort, Essy Behravesh. The downstairs lab would definitely have not been the same without you. Thanks for all the laughs, advice and support you've given me during my tenure at Rice. You truly are a "survivor".

To the Canadian joker, Mark Timmer. Thanks for your friendship and also being one of the black sheep in the downstairs lab. I've enjoyed our discussions as well as playing ball with you. Oh, and congratulations, whenever you decide to get married.

To all my fellow labmates, upstairs and down, past and present. Thank you for all your help and discussions on life, research, and everything else.

To my parents. To my mother, thank you for your prayers and support throughout my sometimes tortuous educational career. To my father, thank you for being a model for my education and upbringing.
To my thesis committee members, Drs. West, Zygourakis, and Anvari. Thank you all for being a part of my graduate education.

To my advisor, Dr. Antonios Mikos. Thank you for providing the opportunity to do research in a world class laboratory. I am truly grateful for your academic and financial support.

Lastly, I would like to dedicate this work to my Heavenly Father. For His eternal gift of salvation, and all the blessings He has bestowed upon me. May He be honored and glorified in all that I do.

"Trust in the LORD with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight." Proverbs 3:5-6
Table of Contents

Abstract................................................................................................................................. ii
Acknowledgements................................................................................................................ iii
Table of Contents ......................................................................................................................... v
List of Figures and Tables ............................................................................................................ viii

Chapter 1 - Introduction ........................................................................................................... 1
  1.1 Overview ......................................................................................................................... 1
  1.2 Vascular Anatomy and Biology ........................................................................................ 1
  1.3 Restenosis: Thrombosis and Neointimal Hyperplasia ....................................................... 3
  1.4 Recent Developments in Stent Technology ...................................................................... 6
  1.5 Hydrogels and Endoluminal Paving ............................................................................... 10
  1.6 Bioactive Polymers ......................................................................................................... 13
  1.7 Poly(propylene fumarate) .............................................................................................. 15
  1.8 Poly(propylene fumarate-co-ethylene glycol) ............................................................... 16

Chapter 2 - Objectives ............................................................................................................. 17

Chapter 3 - Kinetics of Poly(Propylene Fumarate) Synthesis by Step Polymerization of Diethyl Fumarate and Propylene Glycol Using Zinc
  3.1 Introduction ..................................................................................................................... 19
  3.2 Experimental ................................................................................................................... 19
    3.2.1 Materials .................................................................................................................... 19
    3.2.2 PPF Synthesis ............................................................................................................ 20
    3.2.3 Gel Permeation Chromatography ............................................................................ 22
    3.2.4 Calculation of Kinetic Rate Constants .................................................................... 22
    3.2.5 NMR ........................................................................................................................ 25
    3.2.6 FT-IR Spectroscopy ................................................................................................. 26
    3.2.7 Mass Spectrometry ................................................................................................. 26
  3.3 Results and Discussion ...................................................................................................... 26
    3.3.1 Effect of Reaction Time and Temperature on PPF Molecular Weight .................... 26
    3.3.2 Effect of Temperature on PPF Kinetics ................................................................... 34
    3.3.3 Spectral Characterization of Bis(2-Hydroxypropyl) Fumarate and PPF .................. 36
  3.4 Summary .......................................................................................................................... 40
Chapter 4 - Crosslinking Characteristics of and Cell Adhesion to an Injectable Poly(Propylene Fumarate-co-Ethylene Glycol) Hydrogel Using a Water Soluble Crosslinking System ........................................ 41

4.1 Introduction ................................................................................................................. 41
4.2 Experimental .................................................................................................................. 42
  4.2.1 Materials .................................................................................................................. 42
  4.2.2 Poly(Propylene Fumarate) Synthesis ................................................................. 42
  4.2.3 Poly(Propylene Fumarate-co-Ethylene glycol) Synthesis .................. 43
  4.2.4 Hydrogel Fabrication ............................................................................................ 45
  4.2.5 Swelling and Sol Fraction .................................................................................... 45
  4.2.6 Onset of Gelation ................................................................................................. 46
  4.2.7 Tensile Testing ....................................................................................................... 48
  4.2.8 Determination of Molecular Weight between Crosslinks .................. 48
  4.2.9 Cell Adhesion ......................................................................................................... 49
  4.2.10 Statistical Analysis ............................................................................................... 50

4.3 Results .......................................................................................................................... 53
  4.3.1 Swelling .................................................................................................................. 53
  4.3.2 Sol Fraction .............................................................................................................. 53
  4.3.3 Onset of Gelation Time .......................................................................................... 58
  4.3.4 Tensile Testing ....................................................................................................... 58
  4.3.5 Molecular Weight between Crosslinks ............................................................... 58
  4.3.6 Cell Adhesion ......................................................................................................... 63

4.4 Discussion ...................................................................................................................... 63
4.5 Summary ........................................................................................................................ 70

Chapter 5 - Cell Adhesion to Bulk RGD Modified Poly(Propylene Fumarate-co-Ethylene Glycol) Hydrogels ......................................................... 71

5.1 Introduction .................................................................................................................... 71
5.2 Experimental .................................................................................................................. 71
  5.2.1 Materials .................................................................................................................. 71
  5.2.2 Polymer Synthesis ................................................................................................. 72
  5.2.3 Peptide PEG Acrylate Synthesis ............................................................................. 73
  5.2.4 Bulk Peptide Modified Hydrogel Fabrication ...................................................... 73
  5.2.5 Cell Culture ............................................................................................................. 75
  5.2.6 Cell Adhesion ......................................................................................................... 75
  5.2.7 Statistics ................................................................................................................ 76

5.3 Results and Discussion ................................................................................................. 76
5.3 Summary ......................................................................................................................... 81
Chapter 6 - Conclusions ................................................................. 82
References ...................................................................................... 84
List of Figures and Tables

Figure 1-1. Diagram of arterial anatomy. 2

Figure 1-2. Schematic of the events during restenosis. 5

Figure 1-3. A typical stent used after balloon angioplasty. 7

Figure 3-1. Reaction scheme for the step polymerization of poly(propylene fumarate) from propylene glycol and diethyl fumarate. 21

Figure 3-2. Variation of number average degree of polymerization with time with fitted lines for the calculation of the initial rate constants at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). 24

Figure 3-3. Gel permeation chromatographs of PPF polymers at different reaction times at 130°C and the bis(2-hydroxypropyl) fumarate diester intermediate. 28

Figure 3-4. Variation of PPF number average molecular weight with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). 29

Figure 3-5. Variation of PPF weight average molecular weight with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). 30

Figure 3-6. Variation of PPF polydispersity with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). 31

Table 3-1. Initial rate constants for PPF polymerization. 35

Figure 3-7. Desorption chemical ionization mass spectrum of the diester intermediate. 37

Figure 3-8. A representative proton NMR spectrum of purified PPF. 38
Figure 3-9. Representative IR spectra of: (A) the diester intermediate and (B) the purified PPF.

Figure 4-1: Schematic of copolymer hydrogel fabrication.

Figure 4-2. Representative plot of the magnitude of the complex viscosity as a function of time for formulation 16.

Table 4-1: Formulations tested in the Full Factorial Design

Table 4-2: Formulations tested in the Resolution IV, Two Level Fractional Factorial Design.

Figure 4-3. The main effects of the mPEG block length of the P(PF-co-EG) copolymer, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the EWC of the copolymer hydrogels.

Table 4-3: Summary of Results for Equilibrium Water Content, Sol Fraction, Gelation Time, and Cell Adhesion.

Table 4-4: Summary of Results for Tensile Modulus, Tensile Strength, and Molecular Weight between Crosslinks ($M_c$).

Figure 4-4. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the sol fraction of the copolymer hydrogels.

Figure 4-5. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the onset of gelation time of the copolymer hydrogels.

Figure 4-6. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the tensile strength of the copolymer hydrogels.

Figure 4-7. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on
the tensile modulus of the copolymer hydrogels.

Figure 4-8. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the molecular weight between crosslinks ($M_c$) of the copolymer hydrogels.

Figure 4-9. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the % cell adhesion of the copolymer hydrogels.

Figure 5-1. Fabrication of peptide modified P(PF-co-EG)/PEG-DA networks

Figure 5-2. Adhesion of endothelial cells to hydrogels.

Figure 5-3. Adhesion of smooth muscle cells to hydrogels.

Figure 5-4. Adhesion of fibroblasts to hydrogels.
Chapter 1 - Introduction

1.1 Overview

Atherosclerosis, the buildup of atherosclerotic plaque in the arteries affects millions of Americans each year. This cardiovascular disorder can lead to chronic heart disease and eventually, myocardial infarction, which can lead to death. One of the most effective and popular methods of treating atherosclerosis is a procedure known as Percutaneous Transluminal Coronary Angioplasty (PTCA), more commonly known as balloon angioplasty. Over 300,000 such procedures are performed each year in the United States (Califf et al., 1991). In this procedure, a thin hollow catheter is inserted into the coronary artery and a tiny balloon is inflated to restore the blood flow back to the heart. However, in 30-60% of PTCA procedures, retreatment of the patients is necessary due to restenosis or reocclusion of the blood vessel (Bauters and Isner, 1997). It has been estimated that these repeat procedures cost the American health care system over 750 million dollars a year (Bauters and Isner, 1997).

1.2 Vascular Anatomy and Biology

Blood vessels are made up of three distinct layers known as the tunica: the intima, the media, and the adventitia. These three layers all consist of different cell phenotypes and serve different functions for the vessel. The intima is made up of a monolayer of endothelial cells. This monolayer, also called the endothelium, controls many functions, but most importantly, it serves to maintain a non-thrombogenic environment for flowing blood in the cardiovascular system. The endothelium rests on a loose bed of connective
Figure 1-1: Diagram of arterial anatomy
tissue and a thin layer of elastic fibers called the internal elastic lamina. In veins, the internal elastic lamina is absent. The tunica media is made up mostly of smooth muscle cells in a collagen and elastin matrix. In arteries, this layer is much more prominent and contains a greater quantity of smooth muscle fibers. The outer most layer, the tunica adventitia, consists mostly of collagen fibers. This layer also contains fibrous tissue and some fatty tissue. In arteries, a thin elastin layer, known as the external elastic lamina, divides the media and the adventitia. The medial and adventitial layers are perfused with the vaso vasorum, small vessels which provide the blood supply to these outer layers.

1.3 Restenosis: Thrombosis and Neointimal Hyperplasia

Although both balloon angioplasty and metallic stents have been used with relative success in practice, restenosis still occurs and often requires another procedure to open up the occluded vessel. The pathologies most often associated with restenosis are thrombosis and neointimal hyperplasia (Schwartz, 1998). In order to design a better stent, these two problems must be dealt with. To better understand why these problems take place, an in depth look at the mechanisms behind thrombosis and neointimal hyperplasia is required.

Thrombosis is the occlusion of a blood vessel due to the clotting of platelets. This clotting usually occurs in response to injury of the inner lining of the vessel wall or the endothelium. The endothelium consists of a monolayer of endothelial cells which is non-reactive to platelets. However, once this monolayer is disrupted, the exposed non-endothelial surface activates the platelets and causes them to coagulate. During implantation of the graft, platelets may become activated due to the massive release of
factors into the blood from vessel injury. Most important is the thrombogenicity and smoothness of the stent material itself. If the stent material is thrombogenic and rough, platelets and fibrinogen will adhere to the luminal surface of the graft and initiate the coagulation cascade. Total occlusion of the blood vessel may occur.

Neointimal hyperplasia is the occlusion of a blood vessel due to the uncontrolled proliferation of smooth muscle cells, the cells that make up the muscles of the vessel wall. This intimal thickening is again due to injury to the endothelium of the vessel. This injury can be from a number of sources including the implantation of a graft or from balloon angioplasty. The mechanism behind neointimal thickening after balloon angioplasty are still not well understood, however, the initial event in restenosis has been found to be release of fibroblast growth factor and platelet derived growth factor from adhered platelets (Bauters and Isner, 1997). These mitogens cause smooth muscle cells to migrate and proliferate at the injury site. Finally, synthesis of matrix proteins by the smooth muscle cells contribute to the overall neointimal thickening (Figure 1-2).

Consequently, the ideal stent would be one that is completely non-thrombogenic and promotes the proliferation and adhesion of endothelial cells while inhibiting the migration and proliferation of smooth muscle cells.
Figure 1-2: Schematic of the events during restenosis
1.4 Recent Developments in Stent Technology

In order to reduce the occurrences of restenosis after balloon angioplasty, physicians have used stents as a mechanical means of keeping the blood vessel patent (Figure 1-2). The majority of these stents are made of metals such as stainless steel, tantalum or Nitinol (Violaris et al., 1997). However, although these metallic stents have proven to significantly reduce the rates of acute reocclusion, complications from thrombosis and chronic restenosis have still been reported. It has been reported that patients receiving metallic stents have a 20-30% chance of in-stent restenosis (Garas et al., 2001). The main concerns with metallic stents are the inherent properties of the metal used and the permanence of the implant.

Metals, by nature, have a net positive surface potential making them inherently thrombogenic since blood components are negatively charged (Peng et al., 1996). Furthermore, metals are non-degradable. Once a metallic stent has been in put in place, it will forever remain as a foreign body in the blood vessel leading to long-term immune responses. Also, because of the natural stiffness of the metals, compliance mismatch can occur. The difference in mechanical properties between the flexible artery and the stiffness of metals can lead to continual stress at the implantation site leading to neointimal thickening (Stewart and Lyman, 1992).

One of the more promising methods of treating restenosis after PTCA as well as in-stent restenosis has been intravascular brachytherapy, the use of ionizing radiation inside the coronary artery. Both gamma and beta radiation has been shown to significantly reduce neointimal hyperplasia after ballon angioplasty (Garas et al., 2001).
Figure 1-3: A typical stent used after balloon angioplasty
In both cases, the radiation breaks the bonds of single stranded and double stranded DNA in dividing cells, thereby, preventing the proliferation of smooth muscle cells. In clinical trials of intracoronary radiation to treat in-stent restenosis, up to a 60% reduction in recurrence of restenosis was demonstrated (Chan and Moliterno, 2001). Currently, beta radioactive stents coated with phosphorus-32 are being investigated. Although these stents could possibly eliminate in-stent restenosis, problems still do exist. For instance, stent-edge hyperplasia from the stimulating effect of the radiation and balloon injury may occur (Chan and Moliterno, 2001).

Another method that has been used to counteract the effects of the metals used in stents has been to employ a more biocompatible coating. These coatings range from inert metals to polymeric materials. Windecker et al. have coated stainless steel stents with a titanium-nitride-oxide (TiNOX) coating (Windecker et al., 2001). These TiNOX coated stents were shown to significantly reduce neointimal hyperplasia in a porcine model. However, these stents were implanted into normal coronary arteries without injury. Further studies need to be performed in order for this method to be validated.

The other approach is to coat the stent with a polymeric material. The polymer coating can not only reduce the thrombogenicity of the metal stent materials, but can also serve as a carrier for drugs. A variety of polymer coatings have been implemented including polyurethane, silicone, and Nylon (Peng et al., 1996). Fibrin, a natural resorbable polymer, has also been used to coat metallic stents. In comparison to polyurethane coated stents, the fibrin coated stents showed a decrease in neointimal thickening in a porcine coronary artery model (Holmes et al., 1994). Although, this
method looks promising, several issues such as immune response and donor infection still must be resolved. Lambert et al. have studied the release of forskolin from a polyurethane coated stent. Biologically active forskolin was delivered to the arterial wall at a high concentration relative to the blood and other tissues. Also, poly(L-lactic acid) (PLLA) loaded with dexamethasone has been used to coat stents. Studies have shown effective delivery of dexamethasone for up to 28 days, although no significant reduction in restenosis was observed (Lincoff et al., 1997). Other drugs that have been loaded into polymer coated stents include paclitaxel, nitric oxide, and phosphorylcholine (Buergler et al., 2000; Farb et al., 2001; Grenadier et al., 2002). The results of these studies have been mixed thus far. The stents coated with nitric oxide showed severe stenosis while the phosphorylcholine coated stents showed no difference from uncoated stents. Stainless steel stents were coated with chondroitin sulfate and gelatin containing paclitaxel. After 28 days of implantation in rabbits, reduced neointima was seen, however, evidence of incomplete healing was also observed. Although these polymer coated stents show a little promise in delivering biologically active drugs to the arterial wall, more work is required to demonstrate their clinical efficacy.

Thus, in recent years, researchers have been investigating the use of polymers as a potential stent material. The advantages of using polymers are threefold. First, by changing the processing of the polymer, a smooth, non-thrombogenic surface can be produced as well as desired mechanical properties. Secondly, drugs can be incorporated into the polymer matrix for controlled release. Drugs that can prevent thrombosis and neointimal hyperplasia such as heparin and taxol can be delivered at a much higher concentration than by conventional means (Tanguay et al., 1994). Lastly, polymers such
as poly(L-lactic acid) or poly(glycolic acid) can be used that are biodegradable thereby avoiding another surgical procedure to remove the device.

Currently, PLA, PGA, polycaprolactone, and polyethylene teraphthalate (PET) have been the polymers of choice in the development of polymeric stents (Agrawal et al., 1992; Peng et al., 1996). However, each of these polymers has their advantages and drawbacks. For instance, PLA and PGA are polymers that degrade into nontoxic byproducts. Unfortunately, as these materials degrade they also lose their mechanical properties. The biocompatibility of these polymer stents is still a debated issue. It has been found that these polymers invoke an unusually large inflammatory response in a porcine coronary artery model (van der Giessen et al., 1996). On the other hand, a more recent study using a coiled helical poly(L-lactic) acid (PLLA) stent in humans showed minimal inflammatory response after 6 months (Tamai et al., 2000). PET has excellent mechanical properties, but it is non-degradable as well as being thrombogenic. Studies have also shown a significant inflammatory response when PET stents have been tested in a porcine animal model (Murphy et al., 1992). Clearly, more research must be done before polymeric stents can be implemented clinically.

1.5 Hydrogels and Endoluminal Paving

Another area in which research is being done is in the field of hydrogel coatings. A hydrogel is defined as a cross-linked network of hydrophilic polymers that swell in water (Ratner et al., 1996). Hydrogels are a class of polymers that have been shown to be extremely biocompatible materials. Hydrogel materials mimic biological tissues in their physical properties making them an excellent candidate for biomedical applications.
Examples of current hydrogels include poly(ethylene) glycol, poly(vinyl) alcohol and poly(hydroxyethyl) methacrylate.

Poly(ethylene glycol) (PEG) based hydrogels have played an important role in the arena of biomaterials, filling many roles in drug delivery and tissue engineering due to their biocompatibility and excellent physicochemical properties. Free radical initiation has been particularly useful for the synthesis of hydrogels. Hydrogels have been synthesized using ultra-violet (UV) light as well as thermal free radical initiation systems. UV initiation systems have been extensively investigated in biomaterials for orthopedic and cardiovascular applications (Drumheller and Hubbell, 1995; Lu and Anseth, 1999; Nakayama and Matsuda, 1999; Mann et al., 2001; Mellott et al., 2001; Ward and Peppas, 2001). Such initiation systems have incorporated the use of acetophenone, trimethylbenzoacylphosphine oxide (BAPO), and 2,2-dimethoxy-2-phenylacetophenone (DMPA). Thermal initiation systems that have been investigated have utilized such initiators as benzoyl peroxide and azo-bis-(isobutynitrile) (AIBN) (Macret and Hild, 1982; Yeh et al., 1994; Suggs et al., 1998). However, these initiators are insoluble in water and require an organic solvent to dissolve them.

Water soluble redox initiators are another class of thermal initiators that have been examined for hydrogel synthesis. Free radicals can be easily generated under mild conditions, e.g., low temperature, through an electron transfer mechanism. These initiators also have the advantage of being soluble in water circumventing any use of organic solvents. Examples of water soluble redox initiators include a number of persulfates, peroxides, and bisulfites (Sarac, 1999). Redox initiators have been utilized for hydrogels that have been examined for use as absorbents and drug carriers (Huglin et
al., 1997; Liu and Rempel, 1997; Zhou et al., 1997; Gotoh et al., 1998; Mathur et al., 1998; Zhou et al., 1999). The persulfate-ascorbate redox pair is one such water soluble redox pair. In an aqueous environment, the reaction of persulfate and ascorbate ions results in the generation of persulfate and ascorbate radicals, which facilitate the crosslinking of the hydrogel. The rate and extent of the polymerization has been shown to be dependent on the concentration of both oxidizer and reducer (Sarac, 1999).

A variety of crosslinking agents have been used in the synthesis of hydrogels. Examples of crosslinking agents that have been investigated include NVP, PEG dimethacrylate, tri-methoxylpropane triacrylate, and many others (Yeh et al., 1994; Drumheller and Hubbell, 1995; Liu and Rempel, 1997; Iza et al., 1998; Suggs et al., 1998; Ward and Peppas, 2001). PEG-diacrylate (PEG-DA) has also been employed in many hydrogel studies and is suitable as a crosslinking agent for hydrogels due to its water solubility, its hydrophilic properties, and its reactive acrylate bonds (Mann et al., 2001; Mellott et al., 2001; Temenoff et al., 2002).

Sleipan et al. have developed a method where biodegradable hydrogels are coated on to the blood vessel wall via a catheter injection system (Sleipan, 1994; Sleipan, 1996). The hydrogels were crosslinked using an in situ photopolymerization technique. These endoluminal pavings can serve as depots for drugs as well as providing a non-thrombogenic barrier to platelets and mitogenic factors. Hill-West et al. have tested a copolymer of PEG and PLA also employing an in situ photopolymerization system in a rat balloon injury model (Hill-West et al., 1994). Treatment with the PEG-co-PLA hydrogel barrier reduced the amount of intimal thickening by 90%. Hydrogels have also been investigated for use as a coating on to metallic stents to improve biocompatibility as
well as act as a carrier for drugs and viral vectors for gene therapy. Nakayama et al. have photopolymerized a gelatin macromer on to gold stents impregnated with a model drug and an adenoviral vector (Nakayama et al., 2001). After three week implantation in rats, release of drugs and gene expression was observed. In addition, minimal inflammatory response to the gelatin photogel was observed.

1.6 Bioactive Polymers

One of the most recent developments in promoting the growth and adhesion of endothelial cells and reducing thrombogenicity on hydrogels is the incorporation and immobilization of biologically active molecules on the polymer itself. The properties of hydrogels, including large numbers of polar reactive sites, make it particularly suitable for this purpose. One of the most novel areas of research currently being conducted is the incorporation of oligopeptide sequences to enhance endothelial cell adhesion. Oligopeptides are short peptide sequences that have been identified and derived from proteins that are known to mediate cell adhesion. These peptide sequences can be covalently attached to a polymer substrate. In comparison with proteins, these short peptide sequences have the advantage of being much more stable and less susceptible to denaturation (West et al., 1997).

One of the most ubiquitous peptides utilized in the literature has been the GRGD oligopeptide sequence. RGD has been identified as the minimal peptide sequence required for cell-ligand interactions particularly in integrin binding. This sequence has been found in several proteins including fibronectin, vitronectin, and laminin (Ruoslahti and Pierschbacher, 1987). These peptides have been used to modify many different non-
cell adhesive biomaterials (Cook et al., 1997; Hern and Hubbell, 1998; Elbert and Hubbell, 2001; Lin et al., 2001; Mann et al., 2001; Shin et al., 2002). GRGD has been incorporated into a photopolymerizable hydrogel of PEG diacrylate. Adhesion studies showed significantly higher fibroblast attachment and spreading (Hern and Hubbell, 1998). A resorbable polymer of poly(lactic acid-co-lysine) has also been modified with GRGD sequences. After incubation with bovine aortic endothelial cells, these materials showed greater amount of cell spreading than controls (Cook et al., 1997). Hydrogels made from novel oligomers of PEG and fumarate bonds have been functionalized with RGD (Shin et al., 2002). Primary marrow stromal cells were well spread on the functionalized hydrogels. Cell adhesion was shown to be dependent on the peptide concentration and the length of the PEG spacer.

Hubbell et al. have covalently attached GREDVY peptide sequences derived from fibronectin on to PEG coated polyethylene terephthalate (Hubbell et al., 1991; Hubbell et al., 1992). Endothelial cells attached and spread completely on the GREDVY attached substrates while smooth muscles cells and platelets failed to attach. These peptides have also been grafted on to other non-adhesive substrates. Drumheller et al. have bound GREDVY on to the surface of non-cell adhesive matrices made of PEG diacrylate crosslinked with trimethylolpropane triacrylate and acrylic acid (Drumheller and Hubbell, 1994). These peptide-modified substrates supported morphologically complete fibroblast adhesion. YIGSR, an oligopeptide found in laminin, is another sequence that has also been found to promote endothelial cell specific attachment (Massia et al., 1993). When attached to aminophase glass, this peptide sequence showed a significant increase in endothelial cell proliferation when compared to controls (Dee et al., 1995). The YIGSR
and RGD peptide sequences have also been shown to promote and enhance the migration of endothelial cells on to surfaces modified with these peptides (Kouvroukoglou et al., 2000). These results are extremely encouraging in terms of their potential in vascular applications.

1.7 Poly(propylene fumarate)

PPF is an unsaturated, linear polyester that is biodegradable and in situ polymerizable. PPF degrades into propylene glycol and fumaric acid, which are non-toxic byproducts that can be easily passed through the body (He et al., 2001). It can be crosslinked at the time of surgery to form a solid degradable bone cement via an addition polymerization with N-vinyl pyrrolidone (N-VP). As the crosslinking reaction proceeds, the PPF is transformed from a viscous liquid to a putty-like state before finally solidifying. During its liquid and putty states, the cement can be injected or molded into the bone defect. It is therefore well suited for this application since many bone injuries result in defects, which are relatively inaccessible without further surgical exposure and geometrically ill defined. Most addition polymerization reactions are exothermic and generate large quantities of heat. In the case of PMMA, which is polymerized in situ, this is sufficient to cause some local tissue necrosis. In contrast, much less heat is generated by the crosslinking reaction between PPF and N-VP (Peter et al., 1999) and no local tissue necrosis has been noted in in vivo studies (Yaszemski et al., 1995). The mechanical properties of the crosslinked PPF incorporated with tricalcium phosphate have also been found to be comparable to that of trabecular bone (Peter et al., 1999).
1.8 Poly(propylene fumarate-co-ethylene glycol)

Suggs et al. have developed a biodegradable copolymer of PPF and PEG for use in cardiovascular applications and endoluminal paving (Suggs et al., 1998). The PPF and PEG are transesterified at high vacuum and high temperature to produce block copolymers of PPF and PEG. These copolymers can also be crosslinked using N-vinyl pyrrolidone (N-VP) as a crosslinking agent. The PPF provides mechanical strength and crosslinkable sites while the PEG provides the hydrophilic properties required in cardiovascular applications. The copolymers were found to be either diblock or triblock in structure. In vitro degradation studies showed that over a period of 12 weeks, copolymers with 50% and 25% PEG crosslinked with NVP lost 60% of their original mass (Suggs et al., 1998). In vivo biocompatibility tests using a 21 day cage implant system showed excellent biocompatibility signified by the absence of foreign body giant cells (Suggs et al., 1999). These copolymers have also shown to have the capability to act as carrier for endothelial cells in cell transplantation applications. Endothelial cells demonstrated the ability to proliferate even while embedded in the copolymer matrix (Suggs and Mikos, 1999).
Chapter 2 - Objectives

The ultimate goal of the project was to develop an injectable material that could prevent restenosis and thrombosis after balloon angioplasty. In order for such a material to be feasible, it must satisfy certain criteria. A suitable material should be non-cell adhesive so that fibroblasts and smooth muscle cells will not adhere and initiate restenosis. The material must also be in situ crosslinkable in order for it to harden at the site of angioplasty. In addition, the material should have sufficient mechanical strength to withstand the physiological forces inside the blood vessel.

To accomplish these goals, we proposed that an injectable hydrogel formulated from a copolymer of poly(propylene fumarate) (PPF) and methoxy poly(ethylene glycol) (mPEG) may be suitable for such an application. PPF is a material that has been extensively characterized in our laboratory. However, few studies have dealt with the reaction kinetics of synthesizing PPF. An important part of this project was the ability to synthesize PPF with a consistent molecular weight. Therefore, the first part of this project was to characterize the kinetics of the PPF synthesis. The molecular weight of PPF was monitored over 12 hours at three different temperatures. Utilizing this data, the initial rate constants were also calculated.

Once the kinetics of the PPF reaction had been properly characterized, copolymers of PPF and mPEG were synthesized. Suggs et al. had previously synthesized copolymers with PPF and PEG. Our proposed approach utilized mPEG in order to create specifically tri-block copolymers with PEG chains flanking the PPF block. Furthermore, a novel, water soluble crosslinking system was devised to preclude the use of organic
solvents. This crosslinking system consists of the redox pair, ammonium persulfate and ascorbic acid, and the crosslinking monomer, PEG diacrylate. Thus, the second part of this project was to thoroughly characterize hydrogels fabricated with this crosslinking system and the tri-block copolymers. The effects of four different factors on several different properties were studied using a factorial experimental design. The following properties were investigated: equilibrium water content, sol fraction, mechanical strength, onset of gelation, molecular weight between crosslinks, and endothelial cell adhesion. The aims of this study were not only to assess which factors were important in modulating the properties, but also to examine the suitability of the material for cardiovascular applications.

One of the advantages of our material is that due to the presence of its many double bonds, it can be functionalized with bioactive peptides to promote the adhesion and migration of cells. In this project, the promotion of adhesion and migration of endothelial cells to regenerate the endothelial lining after angioplasty would be exceptionally useful. To test the possibility of bioactive functionalization of the copolymer hydrogels, the RGD peptide sequence was covalently incorporated into the hydrogel network. To assess the bioactivity of the peptides, the adhesion of three cell types: endothelial cells, smooth muscle cells, and fibroblasts was tested.
Chapter 3

Kinetics of Poly(Propylene Fumarate) Synthesis by Step Polymerization of Diethyl Fumarate and Propylene Glycol Using Zinc Chloride as a Catalyst

3.1 Introduction

As mentioned in the preceding chapter, control of the molecular weight of PPF is an important part of the project. The molecular weight of linear PPF has been shown to affect the mechanical and degradative properties of a crosslinked composite for orthopedic applications (Domb et al., 1990; Yaszemski et al., 1995; Yaszemski et al., 1996; Peter et al., 1997; Peter et al., 1997; Peter et al., 1998; Peter et al., 1999). In order to synthesize polymers with a reproducible molecular weight, control of the polymer synthesis as well as an understanding of the reaction kinetics are required. Although many different methods for synthesizing PPF have been reported (Gerhart et al., 1988; Domb et al., 1990; Gresser et al., 1995; Kharas et al., 1997; Peter et al., 1997; Peter et al., 1999), few publications have dealt with the reaction kinetics (Peter et al., 1997). The objectives of this study were first to characterize the kinetics of PPF formation by monitoring molecular weight over time and then to determine the effect of temperature on the polymerization kinetics.

3.2 Experimental

3.2.1 Materials

Diethyl fumarate (Acros, Pittsburgh, PA) and propylene glycol (Acros) were used as received. Hydroquinone (Aldrich, Milwaukee, WI) and zinc chloride (Aldrich) were
also used as received. All solvents were purchased from Aldrich as reagent grade and used as received.

3.2.2 PPF Synthesis

The PPF synthesis method was adapted from Kharas et al. (Kharas et al., 1997). Diethyl fumarate and propylene glycol were added to a three-necked round bottom flask in a molar ratio of 1:3, respectively. In addition, hydroquinone was added as a crosslinking inhibitor and zinc chloride was added as a catalyst, in a 0.002:0.01:1 molar ratio to diethyl fumarate, respectively. The solution was maintained under nitrogen, submerged in an oil bath, and mechanically mixed using an overhead stirrer. The initial temperature was set at 100°C and gradually raised to 150°C in 10⁰ increments over a period of one hour. Ethanol, a byproduct of the reaction, was collected as a distillate. The reaction was run until 90% of the theoretically expected ethanol was received.

After removal of the ethanol, the solution was allowed to cool to 100°C and then placed under vacuum (<1 mmHg). The temperature was increased in 10⁰ increments every 15 minutes up to the reaction temperature for the 130°C and 150°C runs to avoid bumping. For the polymerization at 200°C, the temperature was raised in 15°C increments every 15 minutes. Propylene glycol was driven off and collected as a distillate during the self- transesterification of the diester intermediate, bis(2-hydroxypropyl) fumarate (Figure 3-1). The reaction was run for 12 hours after the application of the vacuum, and samples were collected each hour for GPC analysis. The resulting polymer was dissolved in methylene chloride. The reaction was repeated three times for each polymerization temperature tested.
Figure 3-1. Reaction scheme for the step polymerization of poly(propylene fumarate) from propylene glycol and diethyl fumarate.
For purification, a 5% v/v solution of 1 N HCl was added to the CH₂Cl₂ solution of polymer in a separatory funnel to remove the zinc chloride. The product was then washed in a similar fashion with double distilled H₂O and brine. After drying with sodium sulfate, the polymer was concentrated by rotoevaporation and then precipitated in ethyl ether under mechanical stirring to remove the hydroquinone inhibitor. The ethyl ether was decanted and the purified polymer was vacuum dried to remove any residual solvent.

3.2.3 Gel Permeation Chromatography

Gel permeation chromatography (GPC) was employed to determine the PPF molecular weight distribution at each time point. A Styragel guard column (50 x 7.8 mm, mixed bed, Waters, Milford, MA) and a Styragel HR2 GPC column (300 x 7.8 mm, mixed bed, Waters) were used to elute the samples in chloroform at 1 mL/min. A differential refractometer detector (Waters, Model 410) was utilized to obtain the molecular weight distribution of the polymers. Polystyrene standards (Polysciences) of Mₙ 500, 2630, 5970, and 9100 were chosen to generate calibration curves.

3.2.4 Calculation of Kinetic Rate Constants

The initial rate constants were calculated for the PPF polymerization reactions done at 130°C, 150°C and 200°C. Although the transesterification is reversible, the reverse reaction can be neglected especially since the propylene glycol is being driven off. Assuming that the transesterification reaction produces propylene glycol as the only byproduct, the rate equation can then be written as:
\[-\frac{d[M]}{dt} = k[M]^2\] (1)

Where \([M]\) is the concentration of end hydroxyl groups in PPF and \(k\) is the initial rate constant (Odian, 1991). This expression can be integrated and rearranged to yield:

\[\bar{X}_n = 1 + [M]_0 kt\] (2)

Where \(\bar{X}_n\) is the number-average degree of polymerization and \([M]_0\) is the initial hydroxyl group concentration. \(\bar{X}_n\) was calculated by dividing the \(M_n\) of the PPF at each time point by the molecular weight of the repeating unit. Its molecular weight was determined from 2-hydroxypropyl maleate, an isomer of the repeating unit. The isomer was synthesized by the reaction of maleic anhydride and propylene glycol using a previously published method (Domb et al., 1990). The number average molecular weight of 2-hydroxypropyl maleate was determined by GPC using polystyrene standards since the \(M_n\) of the PPF polymers were also determined using the same method. The \(M_n\) of 2-hydroxypropyl maleate and hence the repeating unit was found to be 370.

The initial rate constants were determined from GPC data from 1 hour to 4 hours. Data were used starting from 1 hour to take into account the time to reach the reaction temperature as well as removal of the excess propylene glycol. After 4 hours, it was assumed that the reaction would slow down due to diffusional limitations from the increased viscosity of the mixture and the decreased ability to remove the propylene glycol at 130°C and 150°C. In the case of the transesterification at 200°C, the polymer
Figure 3-2. Variation of number average degree of polymerization with time with fitted lines for the calculation of the initial rate constants at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). Error bars represent means ± standard deviations for n=3.
crosslinked after 4 hours; therefore, the initial rate data from 1 to 3 h were utilized to calculate the rate constant.

Lines were fitted to plots of $\bar{X}_n$ vs. time and the slopes of the lines were calculated (Figure 3-2). According to equation 2, the slope was equal to $[M]_0 k$. In order to find the rate constant, $k$, the initial hydroxyl concentration, $[M]_0$, was determined. Although the initial reaction solution at 1 hour may have contained a small amount of propylene glycol and higher molecular weight oligomers other than the diester, the assumption was made that the initial solution consisted completely of bis(2-hydroxypropyl) fumarate. To find $[M]_0$, the molar concentration of the monomer needed to be determined. To do this, the density of the diester intermediate was found by measuring the mass of a known volume of bis(2-hydroxypropyl) fumarate which was synthesized according to a previously published method (Domb et al., 1990). The initial monomer concentration was calculated by dividing the density of the bis(2-hydroxypropyl) fumarate by its theoretical molecular weight (232). Since there are two hydroxyl groups per diester, $[M]_0$ was determined by multiplying the monomer concentration by two. Since the initial hydroxyl concentration was known, the initial rate constants could be calculated.

3.2.5 NMR

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker 400 MHz NMR (Switzerland) spectrometer. $^1$H spectra were obtained using polymer solutions in
CDCl₃ at ambient temperature. Chemical shifts were presented in reference to TMS. ¹H spectra were taken using standard pulse programs with a three second relaxation delay.

3.2.6 FT-IR Spectroscopy

Fourier transform infrared (FT-IR) spectra were obtained on a Nicolet 500 spectrometer (Madison, WI). Samples were dissolved in CDCl₃ and placed on a calcium fluoride window (Aldrich, Milwaukee, WI). After forming a thin film by evaporating the solvent with nitrogen gas, sixteen scans were collected at a 4 cm⁻¹ resolution by using the calcium fluoride window as a reference.

3.2.7 Mass Spectrometry

The mass spectrum (MS) of the bis(2-hydroxypropyl) fumarate intermediate was obtained in desorption chemical ionization (DCI) mode on a Thermo Finnigan MAT 95 (San Jose, CA) spectrometer. Methane was used as the reagent gas.

3.3 Results and Discussion

3.3.1 Effect of Reaction Time and Temperature on PPF Molecular Weight

Molecular weight data obtained from GPC were used to monitor the progress of the polymerization over time. As the reaction proceeds, the molecular weight of the PPF should gradually increase with time. The first step of the PPF reaction is oligomerization of the diethyl fumarate and propylene glycol by transesterification, which produces ethanol as a byproduct (Figure 3-1). The number average molecular weight of the bis(2-hydroxypropyl) fumarate intermediate as determined by GPC was 515 (±50). This value
is high when compared to its actual molecular weight of 232, and may be attributed to the use of a relative calibration curve with polystyrene standards. The bis(2-hydroxypropyl)fumarate is then reacted at higher temperatures and under vacuum to begin the step polymerization which removes the propylene glycol and drives the reaction forward. The low molecular weight oligomer undergoes a polycondensation reaction via alcoholysis giving off propylene glycol as a byproduct to form the polyester (Figure 3-1). The reaction was run at three different temperatures: 130°C, 150°C, and 200°C. Running the reaction at low temperature reduces the possibility of crosslinking.

The first reaction was run at 130°C. After 3 hours, a significant amount of oligomer still remained in the reactor, but as the reaction progressed, the molecular weight of the polymer rapidly increased and less of the oligomer was seen in the GPC chromatographs (Figure 3-3). The number average molecular weight ($M_n$) increased up to a molecular weight of 2,500 ($\pm 650$) while the weight average molecular weight ($M_w$) increased up to 5,070 ($\pm 1,540$). For both $M_n$ and $M_w$, the molecular weight showed a gradual increase (Figures 3-4 and 3-5). Throughout the reaction, the polydispersity remained constant at 1.9 (Figure 3-6). At 150°C, the $M_n$ exhibited a similar kinetic profile to that at 130°C. The $M_n$ of the PPF increased up to 6 hours (Figure 3-4). After 6 hours the $M_n$ of the PPF was 3,250 ($\pm 410$). From 6 to 12 hours, the $M_n$ increased at a much slower rate. A similar trend was observed for $M_w$. After 12 hours, the PPF had a $M_n$ of 3,990 ($\pm 390$) and a $M_w$ of 10,150 ($\pm 900$). The polydispersity of the polymer rose slowly for 6 hours and then remained relatively constant. The decreased reaction rate may result for a variety of reasons. One explanation may be that after 6 hours, the majority of the propylene glycol
Figure 3-3. Gel permeation chromatographs of PPF polymers at different reaction times at 130°C and the bis(2-hydroxypropyl) fumarate diester intermediate.
Figure 3-4. Variation of PPF number average molecular weight with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). Error bars represent means ± standard deviations for n=3.
Figure 3-5. Variation of PPF weight average molecular weight with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (△). Error bars represent means ± standard deviations for n=3.
Figure 3-6. Variation of PPF polydispersity with polymerization time at three temperatures: 130°C (□), 150°C (◇), and 200°C (∆). Error bars represent means ± standard deviations for n=3.
has been removed. Another reason may be that the starting material, bis(2-hydroxypropyl) fumarate is getting consumed. In addition, diffusional limitations come into play due to the increased viscosity of the polymer.

The higher reaction temperatures also increased the polydispersity of the produced PPF polymers. At 130°C, the purified PPF had a polydispersity of 1.67, while the PPF synthesized at 150°C, had a polydispersity of 2.28. Increasing the reaction temperature increases the degree of polymerization which in turn raises the polydispersity (Odian, 1991).

All three runs of the 200°C transesterification resulted in spontaneous crosslinking of the PPF. GPC analysis carried out on the polymer prior to gelation demonstrated a $M_n$ of 3,330 (±380) and $M_w$ of 9,830 (±1,490). Over a period of three hours, the polydispersity sharply increased indicating branched polymer formation. The resulting material was insoluble in methylene chloride, providing further indication of crosslinking as well as prohibiting any purification. Even in the presence of hydroquinone, a free radical inhibitor, enough radicals may have been generated to initiate the polyaddition reaction, causing the gelation of the PPF.

After 12 hours, the PPF reacted at 150°C had a $M_n$ of 3,990 (±390) and a $M_w$ of 10,150 (±900). Following purification, the values were 4,600 (±160) and 10,500 (±760), respectively. Similar behavior was observed for the PPF synthesized at 130°C. The purified PPF polymerized at 130°C had a $M_n$ of 3,250 (±760) and a $M_w$ of 5,480 (±1520) compared to 2,500 (±650) and 5,070 (±1,540) after 12 hours, respectively. The polydispersities of the PPF were all significantly lowered after purification. The polydispersities of PPF after 12 hours at 130°C and 150°C were 2.00 and 2.55,
respectively and decreased to 1.67 and 2.28 after purification. This decrease may have been due to the precipitation of the polymer into ethyl ether, which not only dissolved the hydroquinone, but may also have removed the lower molecular weight fraction of PPF and narrowed the molecular weight distribution, thus, decreasing the polydispersity.

Several methods have been investigated for synthesizing PPF. A two step reaction between fumaric acid, propylene oxide, and pyridine was examined (Domb et al., 1990). Propylene glycol with maleic anhydride were employed to form a propylene bis(hydrogen maleate) oligomer which was then utilized to synthesize PPF. This reaction was run for 24 hours at 100°C and resulted in PPF of $M_n=1,220$. The results presented in this study show that molecular weights that are four times higher can be obtained in half the reaction time. Direct esterification of fumaric acid and propylene glycol using a p-toluenesulfonic acid monohydrate catalyst was also used to achieve number average molecular weights of 2,600 (Gresser et al., 1995). Peter et al. reported number average molecular weights of 4,900 after 16 hours of transesterification using an oligomer formed from fumaryl chloride and propylene glycol with potassium carbonate as a proton scavenger (Peter et al., 1999). The results presented in this study are similar to those published by Peter et al. with the exception that the polymerization was run for 12 hours. Moreover, the reaction described in this paper is a one-pot reaction and does not require a lengthy oligomerization with additional work-up. Previous studies with diethyl fumarate and propylene glycol reported PPF synthesized with weight average molecular weights of up to 26,600 and a polydispersity of 1.9 at reaction temperatures of 210°C after 6-8 hours (Kharas et al., 1997). However, we find that gelation occurred at reaction temperatures greater than 200°C after 4 hours even with the addition of a radical inhibitor. The
maximum number average molecular weight achievable after 12 hours of transesterification at 150°C and purification was 4,600 (±190).

3.3.2 Effect of Temperature on PPF Kinetics

The effect of temperature on the kinetics of the PPF polymerization was assessed by carrying out the reaction at three different temperatures. As the reaction temperature was increased, the rate of the polymerization reaction also increased. The rate of polymerization was quantified by calculating the initial rate constants at the tested reaction temperatures. The discrepancy between the GPC molecular weight and the true molecular weight of the diester intermediate mentioned previously did not affect the calculated rate constants because this value was not used in the equation. Results are presented in Table 3-1. The initial rate constant for the polymerization reaction increased by more than two times from 130°C to 150°C. A similar increase is seen when the temperature is raised from 150°C to 200°C. The reason for this increase may be three fold. The main reason for the faster reaction rate is that the transesterification rate increases with temperature. Another factor may be as the temperature is increased, the propylene glycol is removed at a greater rate, thereby further driving the polymerization reaction forward. In addition, the product is less viscous at high temperatures, allowing for better diffusion of the propylene glycol out of the reaction mixture.
Table 3-1. Initial rate constants for PPF polymerization.

<table>
<thead>
<tr>
<th>T(°C)</th>
<th>k(L mol⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>0.0014</td>
</tr>
<tr>
<td>150</td>
<td>0.0031</td>
</tr>
<tr>
<td>200</td>
<td>0.0057</td>
</tr>
</tbody>
</table>
3.3.3 Spectral Characterization of Bis(2-Hydroxypropyl) Fumarate and PPF

The DCI mass spectral data of the bis(2-hydroxypropyl) fumarate intermediate yielded a base peak of m/z 233 (M+H\(^+\)), the molecular ion peak (Figure 3-7). A strong peak at m/z 59 (HOCHCH\(_3\)CH\(_2\)OH - OH) indicated the presence of propylene glycol. Other large peaks were seen at m/z 215.2 (MH\(^+\)-H\(_2\)O), 175.1 (HOCH(CH\(_3\))CH\(_2\)OCOCH=CHCOOH\(_2\)\(^+\)) and 157.1 (MH\(^+\)- HOCHCH\(_3\)CH\(_2\)OH). The presence of higher molecular weight oligomers would be revealed by peaks at 389 (n=2) and 545 (n=3). However, no significant peaks were seen greater than 233, indicating the absence of higher molecular weight oligomers. These findings strongly support the assumption that only the diester intermediate and a small amount of propylene glycol are initially in the reactor at the beginning of the transesterification.

Through \(^1\)H NMR spectra, the bis(2-hydroxypropyl) fumarate and PPF chemical structure were verified. The peak assignments were based on tables found in Silverstein et al. (Silverstein et al., 1997). In the PPF spectra (Figure 3-8), the fumarate olefinic protons exhibited signals at 6.85-6.93 ppm. The signals at 1.15-1.5 ppm were the propyl methyl groups. The secondary propyl methine protons were seen at 4.03-4.17 ppm. Finally, the propyl methylene protons were found at 5.0-5.12 ppm. These signals are in good agreement with previously published data (Kharas et al., 1997; Peter et al., 1999).

The infrared spectra of PPF were compared to previously published data to confirm its structure (Peter et al., 1999). IR spectra were especially valuable for the characterization of the oligomer intermediate and PPF. The IR spectrum of oligomer intermediate showed the following characteristic bands (Figure 3-9): a broad -OH stretch centered at 3400 cm\(^{-1}\).
Figure 3-7. Desorption chemical ionization mass spectrum of the diester intermediate.
Figure 3-8. A representative proton NMR spectrum of purified PPF.
Figure 3-9. Representative IR spectra of: (A) the diester intermediate and (B) the purified PPF.
, ester carbonyl at 1740 cm\(^{-1}\), and -C=C stretch at 1650 cm\(^{-1}\). After transesterification of the intermediate, a noticeable decrease of the -OH band at 3450 cm\(^{-1}\) was observed because of the removal of end propylene glycols. The IR spectral change strongly supports the progress of transesterification. The spectra also corroborate previous characterizations of the PPF polymer (Kharas et al., 1997; Peter et al., 1997; Peter et al., 1999).

3.4 Summary

The kinetics of PPF polymerization by reaction of propylene glycol and diethyl fumarate in the presence of zinc chloride were characterized by use of GPC analysis. The initial rate constants and polydispersity increased with higher reaction temperature. At 130°C and 150°C both number average and weight average molecular weights were found to increase steadily up to 12 hours after the application of vacuum. At 200°C, the PPF crosslinked after 4 hours. PPF of up to number average molecular weight 4,600 with a polydispersity of 2.28 was synthesized after 12 hours at 150°C. Both NMR and FT-IR analysis confirmed the structure of the PPF.
Chapter 4

Crosslinking Characteristics of and Cell Adhesion to an Injectable Poly(Propylene Fumarate-co-Ethylene Glycol) Hydrogel Using a Water Soluble Crosslinking System

4.1 Introduction

In this study, the effects of a water soluble crosslinking system, which incorporates a redox initiation mechanism, on the properties of a novel block copolymer of poly(propylene fumarate) (PPF) and methoxy poly(ethylene glycol) (mPEG) were examined. In this system, PEG diacrylate was used as the crosslinker and ammonium persulfate and ascorbic acid were used as the redox initiation system. Random block copolymers of PPF and PEG have been synthesized for cardiovascular applications before (Suggs et al., 1998). Hydrogels were fabricated with N-vinyl-2-pyrrolidinone (NVP) as a crosslinking agent and benzoyl peroxide and dimethyl toluamide (DMT) as the free radical initiator system (Suggs et al., 1998). NVP served a dual purpose, both as a crosslinker, and a solvent for the initiator benzoyl peroxide.

The effects of mPEG block length of the P(PF-co-EG) block copolymer, persulfate concentration, ascorbate concentration, and PEG-DA:P(PF-co-EG) ratio on the properties of the copolymer hydrogel were evaluated in this study. A factorial experimental design was implemented to efficiently determine how the examined factors affected the onset of gelation time, equilibrium water content, mechanical properties, and endothelial cell adhesion. The objectives of this study were not only to ascertain how each factor affected the hydrogel properties, but also which factors were most important in modulating them.
4.2 Experimental

4.2.1 Materials

Diethyl fumarate (Acros, Pittsburgh, PA), propylene glycol (Acros), and monomethyl ether poly(ethylene glycol) of nominal molecular weights of 750 and 5000 (Aldrich, Milwaukee, WI) were used as received. Ascorbic acid (AA) (Sigma, St. Louis, MO) and ammonium persulfate (APS) (Acros) were used as received. All solvents were purchased from Aldrich as reagent grade. Hydroquinone (Aldrich) and zinc chloride (Aldrich) were also used as received. Poly(ethylene glycol) diacrylate of nominal molecular weight 700 was purchased from Aldrich.

4.2.2 Poly(Propylene Fumarate) Synthesis

PPF was synthesized in a two step reaction as previously described (Shung et al., 2002). Briefly, diethyl fumarate and propylene glycol were added to a three-necked round bottomed flask in a molar ratio of 1:3 respectively. In addition, hydroquinone was added as a crosslinking inhibitor and zinc chloride was added as a catalyst, in a 0.01:0.002:1 molar ratio to diethyl fumarate, respectively. The solution was submerged in an oil bath and mechanically mixed using an overhead stirrer under a nitrogen blanket at a maximum temperature of 150° C. Ethanol, a byproduct of the reaction, was collected as a distillate. The reaction was run until 90% of the theoretically expected ethanol was collected.

After removal of the ethanol, the solution was allowed to cool to 100° C and then placed under vacuum (<1 mmHg). During the transesterification, propylene glycol was driven off and collected as a distillate.
4.2.3 Poly(Propylene Fumarate-co-Ethylene glycol) Synthesis

The block copolymer P(PF-co-EG) was synthesized as previously described (Behravesh et al., 2002). The PPF utilized for each copolymer synthesis came from one reaction. Monomethoxy poly(ethylene glycol) (mPEG) was added to the flask in a 1:2 PPF:mPEG molar ratio. Copolymers were synthesized with mPEG nominal molecular weights of either 750 or 5000. The transesterification reaction for the copolymerization was carried out at a vacuum of less than 1 mmHg, with a maximum reaction temperature of 160°C. The extent of the reaction was monitored using gel permeation chromatography (GPC) by checking for the reduction of the free mPEG peak.

To purify the polymer, the resulting product was dissolved in dichloromethane, filtered through a Buchner funnel, and precipitated into ethyl ether under mechanical stirring. Zinc chloride and hydroquinone were soluble in ethyl ether and thus, were removed in the precipitation process. For a waxy copolymer, the ethyl ether was decanted and rotoevaporated in a round bottom flask under vacuum (less than 5 mmHg) at 30°C to remove all remaining solvents. A powdery copolymer was isolated by filtration through a Buchner funnel and subsequently dried under vacuum at room temperature to remove residual organic solvent.
Figure 4-1: Schematic of copolymer hydrogel fabrication
4.2.4 Hydrogel Fabrication

The copolymer was first dissolved in double distilled water (ddH₂O) with an initial water content of 50 wt%. PEG-DA of nominal molecular weight 700 was added as a crosslinking agent in a weight ratio of either 1:1 or 1:3 with respect to copolymer (Figure 4-1). The prepolymer solution was centrifuged to remove air bubbles. APS was then added to the prepolymer, mixed, and centrifuged at concentrations of either 0.1 M or 0.01 M. Finally, the ascorbic acid (AA) was added to the mixture at concentrations of either 0.1 M or 0.01 M and cast in between two glass plates. The copolymer solution was left overnight at 37°C to crosslink. The resulting hydrogel was swollen in phosphate buffered saline (PBS) (GibcoBRL, Grand Island, NY) for 24 hours to reach equilibrium.

4.2.5 Swelling and Sol Fraction

Hydrogel films, equilibrium swollen in PBS, as previously described, were cut into 21 mm diameter disks. The swollen disks were weighed, dried under vacuum overnight, and the resulting weights recorded as the weight of the dry hydrogel. Equilibrium water content was calculated according to the following equation:

\[
% EWC = \left(\frac{W_s - W_d}{W_s}\right) \times 100
\]

where EWC is the equilibrium water content, \(W_s\) is the weight of the equilibrium swollen hydrogel, and \(W_d\) is the weight of the dried hydrogel.
To measure sol fraction, films were fabricated in the same fashion. The films were cut into 5 mm diameter disks, air dried overnight and then dried under vacuum for 24 hours. The dried films were weighed and recorded as the initial dry weight of the film. The disks were then swollen in ddH₂O for 24 hrs with gentle agitation. After swelling, the films were air dried again overnight and then dried under vacuum. The weights recorded were the final dry weights of the films. Sol fraction was calculated according to the following equation:

\[
\% \text{Sol fraction} = \frac{(W_i - W_f)}{W_i} \times 100
\]

where \(W_i\) is the initial dry weight of the hydrogel and \(W_f\) is the final dry weight of the hydrogel.

4.2.6 Onset of Gelation

The onset of gelation was assessed by measuring the change in the complex viscosity after free radical initiation using a rheometer (Model AR1000, TA Instruments, New Castle, DE). Gelation corresponds to the formation of a material where the polymer chains are crosslinked together forming one macroscopic molecule (Sperling, 1992). For this study, the onset of gelation was defined as the time where a sudden increase in the magnitude of the complex viscosity was observed (Figure 4-2) (Peter et al., 1999).

The copolymer and PEG-DA were dissolved in water and APS was added. The solution was well mixed using a vortexer. At time zero, AA was added to the solution, mixed with a vortexer, and placed in a Teflon mold (10 mm in diameter and 15 mm in depth) on top of the rheometer. The temperature controlled rheometer top was set at 37°C. A stainless steel cylinder (8 mm in diameter) was lowered approximately 2 mm
Figure 4-2. Representative plot of the magnitude of the complex viscosity as a function of time for formulation 16. Onset of gelation was defined as the point where there was an abrupt increase in complex viscosity.
into the polymer solution. An oscillatory program was executed consisting of a time sweep with a constant stress of 1000 Pa and a frequency of 1 Hz. The complex viscosity of the crosslinking polymer was measured for approximately 20 min, until a clear change in the complex viscosity was observed.

4.2.7 Tensile Testing

Hydrogel films were again fabricated using the previously described method and cut into dogbone shapes using a stainless steel cutter according to ASTM D638-98 standards. To insure good gripping of the samples, glass slides were attached to the end of the dogbones using cyanoacrylate adhesive. The samples' gauge length was 33 mm and had a thickness of 1 mm. Samples were tensile tested until failure on an Instron 5500 series tabletop load frame (Instron Corporation, Canton, MA) with a 50 N load cell at a cross-head speed of 10 mm/min. The stress and strain data were recorded. Five samples were tested for each formulation. Tensile modulus was calculated by measuring the slope of the linear portion of the stress-strain curve.

4.2.8 Determination of Molecular Weight between Crosslinks

The molecular weight between crosslinks ($M_c$) was determined using both swelling and the tensile modulus data. Assuming affine deformation and small strains, the equation used to approximate the $M_c$ goes as follows (Sperling, 1992):

$$M_c = \frac{1}{3 \rho P RT \varphi^3} \frac{1}{E}$$
where \( R \) is the gas constant, \( T \) is the absolute temperature, \( E \) is the tensile modulus, \( \rho_p \) is the polymer density, and \( \varphi \) is the equilibrium volume fraction of polymer in the swollen state. The equilibrium volume fraction of polymer in the swollen state, \( \varphi \), was calculated using a method described in Peppas and Barr-Howell (Peppas and Barr-Howell, 1986).

\[
\varphi = \frac{V_p}{V_{g,s}}
\]

\[
V_p = \frac{W_{a,d}}{\rho_p}
\]

\[
V_{g,s} = \frac{W_{a,s} - W_{n,s}}{\rho_n}
\]

where \( W_{a,d} \) is the weight of the dried hydrogel sample in air, \( W_{a,s} \) is the weight of the swollen hydrogel in air, \( W_{n,s} \) is the weight of the swollen hydrogel in cyclohexane, \( \rho_n \) is the density of cyclohexane (0.79 g/cm\(^3\)), and \( \rho_p \) is the density of the polymer. All samples were swollen in ddH\(_2\)O and weighed using a hanging pan balance. The density of the polymer, \( \rho_p \), was calculated using the following equation:

\[
\rho_p = \frac{W_{a,d}}{W_{a,d} - W_{n,d}} \rho_n
\]

where \( W_{n,d} \) is weight of the dried hydrogel sample in cyclohexane.

4.2.9 Cell Adhesion

Human umbilical vein endothelial cells (HUVECs) were obtained from American Tissue Culture Company (ATCC, Manassas, VA) and maintained in Ham’s F-12K medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-
Product, Calabasas, CA), heparin (Sigma), and endothelial cell growth supplement (Sigma) at 37°C. Cells of passage 33 were used for the cell adhesion experiment.

The HUVECs were rinsed with PBS and then trypsined for 10 minutes. The cells were then resuspended in 10 mL of medium and centrifuged at 1500 rpm. After aspirating the medium, the cells were resuspended at a concentration of 56,400 cells/mL.

The hydrogel films were cut into 21 mm diameter disks using a cork borer. The films were dipped in 70% ethanol and then soaked in sterile PBS for 24 hours under exposure to UV light. The disks were placed at the bottom of 12 well tissue culture plates. Annular stainless steel rings with inner diameter of 15.5 mm were used to hold the films in place. The stainless steel rings were sterilized by autoclave prior to the experiment. The cells were seeded onto the hydrogels by pipetting 1 mL of the cell suspension into the wells for a seeding density of 30,000 cells/cm². After 8 hours, the wells were rinsed with PBS and the cells were trypsined and counted using a Coulter Multisizer 3 (Beckman Coulter, Fullerton, CA).

4.2.10 Statistical Analysis

A full 2⁴ factorial design was used to assess the effects of four parameters: mPEG block length of the copolymer, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) ratio on the onset of gelation time, equilibrium water content, and sol fraction. This factorial design resulted in sixteen hydrogel formulations being tested (Table 4-1). A resolution IV, 2⁴⁻¹ fractional factorial design was utilized to assess the effects of the four parameters on tensile strength and modulus calling for a total of eight different formulations to be tested (Table 4-2).
<table>
<thead>
<tr>
<th>Level</th>
<th>PEG Molecular Weight</th>
<th>APS Concentration</th>
<th>AA Concentration</th>
<th>PEG-DA/Copolymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>5000</td>
<td>0.1M</td>
<td>0.1M</td>
<td>1:1</td>
</tr>
<tr>
<td>Low</td>
<td>750</td>
<td>0.01M</td>
<td>0.01M</td>
<td>1:3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PEG Molecular Weight</th>
<th>APS Concentration</th>
<th>AA Concentration</th>
<th>PEG-DA/Copolymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4-1: Formulations tested in the Full Factorial Design
<table>
<thead>
<tr>
<th>Level</th>
<th>mPEG Molecular Weight</th>
<th>APS Concentration</th>
<th>AA Concentration</th>
<th>PEG-DA/ Copolymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (+)</td>
<td>5000</td>
<td>0.1M</td>
<td>0.1M</td>
<td>1:1</td>
</tr>
<tr>
<td>Low (-)</td>
<td>750</td>
<td>0.01M</td>
<td>0.01M</td>
<td>1:3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Formulation</th>
<th>mPEG Molecular Weight</th>
<th>APS Concentration</th>
<th>AA Concentration</th>
<th>PEG-DA/ Copolymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4-2: Formulations tested in the Resolution IV, Two Level Fractional Factorial Design
4.3 Results

4.3.1 Swelling

The equilibrium water content of the hydrogels ranged from 57.1±0.3% to 79.7±0.2%. Hydrogels fabricated with a high mPEG block molecular weight of 5000 and low PEG-DA:P(PF-co-EG) ratio of 1:3 showed the greatest amount of swelling. The equilibrium water content values of the swelling studies for all formulations can be found in Table 4-3. The full factorial analysis revealed that all four parameters tested had significant effects on hydrogel swelling (Figure 4-3). The two largest effects involved the mPEG block length of the P(PF-co-EG) and PEG-DA:P(PF-co-EG) ratio. Increasing the mPEG block length in the copolymer increased the equilibrium water content of the hydrogels. However, increasing the PEG-DA concentration decreased the equilibrium water content. APS and AA concentrations also had small effects on swelling. Increasing the APS concentration decreased the equilibrium water content while AA concentration served to slightly increase the equilibrium water content.

4.3.2 Sol Fraction

The sol fraction of the hydrogels ranged from 2.5±0.0% to 33.3±5.4%. The sol fractions of all the hydrogels can be found in Table 4-3. The full factorial analysis showed that only three of the parameters had significant effects on sol fraction: PEG-DA:P(PF-co-EG) weight ratio, APS, and AA concentration (Figure 4-4). AA concentration had the greatest effect on sol fraction. The PEG-DA:P(PF-co-EG) ratio also had a significant effect on the sol fraction. Additionally, an increase in APS concentration caused an increase in the sol fraction of the hydrogels.
Figure 4-3. The main effects of the mPEG block length of the P(PF-co-EG) copolymer, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the EWC of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the EWC from the low level to the high level. A negative number indicates a decrease in the EWC from the low level to the high level. Error bars represent the standard error of the effect.
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Equilibrium Water Content (%)</th>
<th>Sol Fraction (%)</th>
<th>Onset of Gelation (min)</th>
<th>Cell Adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>69.4±0.3</td>
<td>17.6±0.2</td>
<td>1.7±0.2</td>
<td>8.8±7.4</td>
</tr>
<tr>
<td>2</td>
<td>69.8±0.4</td>
<td>7.1±1.8</td>
<td>1.4±0.1</td>
<td>5.8±2.6</td>
</tr>
<tr>
<td>3</td>
<td>69.6±0.2</td>
<td>12.4±1.7</td>
<td>2.4±0.1</td>
<td>8.4±2.6</td>
</tr>
<tr>
<td>4</td>
<td>68.4±0.1</td>
<td>2.9±0.3</td>
<td>2.2±0.1</td>
<td>8.8±3.0</td>
</tr>
<tr>
<td>5</td>
<td>78.1±0.1</td>
<td>22.5±0.4</td>
<td>1.1±0.1</td>
<td>9.6±2.1</td>
</tr>
<tr>
<td>6</td>
<td>79.7±0.2</td>
<td>11.7±2.5</td>
<td>1.1±0.0</td>
<td>3.9±1.4</td>
</tr>
<tr>
<td>7</td>
<td>78.1±0.1</td>
<td>12.3±0.8</td>
<td>1.5±0.1</td>
<td>4.5±2.3</td>
</tr>
<tr>
<td>8</td>
<td>78.2±0.1</td>
<td>4.1±0.5</td>
<td>1.6±0.2</td>
<td>5.4±2.4</td>
</tr>
<tr>
<td>9</td>
<td>57.1±0.3</td>
<td>11.6±5</td>
<td>1.6±0.0</td>
<td>31.1±14.1</td>
</tr>
<tr>
<td>10</td>
<td>60.0±0.2</td>
<td>10.5±0.9</td>
<td>2.6±0.5</td>
<td>9.1±1.4</td>
</tr>
<tr>
<td>11</td>
<td>56.2±0.1</td>
<td>7.4±0.3</td>
<td>1.5±0.2</td>
<td>18.2±3.9</td>
</tr>
<tr>
<td>12</td>
<td>56.2±0.1</td>
<td>2.5±0.0</td>
<td>2.5±0.3</td>
<td>14.6±6.9</td>
</tr>
<tr>
<td>13</td>
<td>58.2±0.3</td>
<td>11.6±0.4</td>
<td>2.2±0.1</td>
<td>23.1±7.4</td>
</tr>
<tr>
<td>14</td>
<td>68.6±0.4</td>
<td>33.3±5.4</td>
<td>4.3±0.2</td>
<td>8.2±9.7</td>
</tr>
<tr>
<td>15</td>
<td>59.5±0.3</td>
<td>6.3±1.5</td>
<td>2.0±0.1</td>
<td>10.6±4.0</td>
</tr>
<tr>
<td>16</td>
<td>60.6±0.2</td>
<td>4.0±0.5</td>
<td>3.0±0.2</td>
<td>8.6±8.3</td>
</tr>
</tbody>
</table>

Table 4-3: Summary of Results for Equilibrium Water Content, Sol Fraction, Gelation Time, and Cell Adhesion
<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tensile Modulus (MPa)</th>
<th>Tensile Strength (kPa)</th>
<th>M_c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4±0.2</td>
<td>173.7±28.2</td>
<td>3580±440</td>
</tr>
<tr>
<td>4</td>
<td>2.2±0.1</td>
<td>261.6±21.6</td>
<td>2380±90</td>
</tr>
<tr>
<td>6</td>
<td>0.4±0.0</td>
<td>64.3±10.7</td>
<td>10950±1110</td>
</tr>
<tr>
<td>7</td>
<td>0.5±0.1</td>
<td>61.7±18.2</td>
<td>9000±1150</td>
</tr>
<tr>
<td>10</td>
<td>2.5±1.0</td>
<td>294.2±61.1</td>
<td>2450±680</td>
</tr>
<tr>
<td>11</td>
<td>3.3±0.3</td>
<td>401.3±67.5</td>
<td>1850±200</td>
</tr>
<tr>
<td>13</td>
<td>2.2±0.4</td>
<td>127.4±17.6</td>
<td>2680±460</td>
</tr>
<tr>
<td>16</td>
<td>1.0±0.3</td>
<td>86.5±26.2</td>
<td>4710±450</td>
</tr>
</tbody>
</table>

Table 4-4: Summary of Results for Tensile Modulus, Tensile Strength, and Molecular Weight between Crosslinks (M_c)
Figure 4-4. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the sol fraction of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the sol fraction from the low level to the high level. A negative number indicates a decrease in the sol fraction from the low level to the high level. Error bars represent the standard error of the effect.
4.3.3 Onset of Gelation Time

A representative plot of the complex viscosity measured by the rheometer showed a distinct increase in viscosity defined as the onset of gelation (Figure 4-2). The onset of gelation times were measured to be between 1.1±0.1 to 4.3±0.2 min (Table 4-3). The full factorial analysis showed that mPEG block length of the copolymer and APS concentration had a significant effect on the onset of gelation time of the resulting hydrogel (Figure 4-5). The mPEG block length had the greatest effect on the onset of gelation time.

4.3.4 Tensile Testing

Mechanical testing of the hydrogels revealed tensile strengths between 61.7±18.2 kPa to 401.3±67.5 kPa and tensile moduli ranging from 0.4±0.0 MPa to 3.3±0.3 MPa. The values for all tensile moduli and strengths of the hydrogels can be found in Table 4-4. A fractional factorial analysis showed that mPEG block length of the P(PF-co-EG) block copolymer and PEG-DA: P(PF-co-EG) ratio had the greatest effects on tensile strength and modulus (Figures 4-6 and 4-7). APS and AA concentration also had significant effects on tensile strength and modulus although not to the same extent as the mPEG block length and PEG-DA:P(PF-co-EG) ratio.

4.3.5 Molecular Weight between Crosslinks

The molecular weight between crosslinks was calculated using both the swelling and tensile modulus data. Results can be found in Table 4-4. The $M_c$ ranged from a maximum of 10950±1110 to a minimum of 1850±200. Results of the fractional factorial
Figure 4-5. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the onset of gelation time of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the onset of gelation time from the low level to the high level. A negative number indicates a decrease in the onset of gelation time from the low level to the high level. Error bars represent the standard error of the effect.
Figure 4-6. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the tensile strength of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the tensile strength from the low level to the high level. A negative number indicates a decrease in the tensile strength from the low level to the high level. Error bars represent the standard error of the effect.
Figure 4-7. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the tensile modulus of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the tensile modulus from the low level to the high level. A negative number indicates a decrease in the tensile modulus from the low level to the high level. Error bars represent the standard error of the effect.
Figure 4-8. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the molecular weight between crosslinks ($M_c$) of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the $M_c$ from the low level to the high level. A negative number indicates a decrease in the $M_c$ from the low level to the high level. Error bars represent the standard error of the effect.
analysis can be found in Figure 4-8. The fractional factorial design showed that the mPEG block length and PEG-DA:P(PF-co-EG) ratio had the greatest effect on $M_c$ compared to the AA and APS concentration. Neither the AA or APS concentration had significant effects on the $M_c$.

4.3.6 Cell Adhesion

A full factorial experiment was done to assess the effect of the factors studied on endothelial cell adhesion. Endothelial cells adhered to the hydrogels from a maximum of 31.1±14.1% to a minimum of 4.5±2.3% of cells seeded. The complete results of the cell adhesion studies can be found in Table 4-3. Out of all the parameters, the PEG block length of the P(PF-co-EG) incorporated had the greatest effect on cell adhesion of all the parameters. Increasing the PEG-DA: copolymer ratio also increased the number of cells adhered (Figure 4-9).

4.4 Discussion

The purpose of this study was to investigate the effects of several factors which influence hydrogel structure and the resulting properties. A factorial design was implemented to efficiently assess which effects of these factors had the greatest impact on the properties studied. Swelling was the first property examined. The mPEG block length of P(PF-co-EG) had the greatest effect on swelling of the hydrogel. With increasing mPEG block length in the copolymer, the molecular weight between crosslinks was greater, allowing more water to diffuse into the hydrogel matrix and, thus,
Figure 4-9. The main effects of the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio on the % cell adhesion of the copolymer hydrogels. A positive number indicates that the particular parameter had an increasing effect on the % cell adhesion from the low level to the high level. A negative number indicates a decrease in the % cell adhesion from the low level to the high level. Error bars represent the standard error of the effect.
a greater equilibrium water content. The PEG-DA: copolymer ratio also had a large effect on equilibrium water content. With increasing PEG-DA: copolymer ratio, more crosslinks were formed with the short chained PEG diacrylate allowing less water to enter and thus decreased swelling was observed.

A small increase in the equilibrium water content was observed when the AA concentration was increased. This increase in equilibrium water content may be because the reduction in pH of the prepolymer solution reduces the effectiveness of the redox initiation reaction and the production of the ascorbate radicals (Sarac, 1999). The decrease in equilibrium water content from the increased APS concentration may be attributed to the greater number of crosslinks caused by the formation of more radicals. The swelling results were typical of highly crosslinked PEG hydrogels (Suggs et al., 1998; Mellott et al., 2001). The amount of uncrosslinked polymer or sol fraction was examined as an indicator of the extent of crosslinking. The sol fraction of the hydrogels ranged from 2.5±0.0% to 33.3±5.4%. AA concentration had the greatest effect on sol fraction. As noted before, an increase in AA concentration may have decreased pH of the prepolymer solution resulting in a decrease in the effectiveness of the redox reaction and the production of the ascorbate radicals. The reduction in the number of free radicals may have decreased the efficiency of the crosslinking reaction resulting in an increase in the amount of uncrosslinked material.

The PEG-DA: copolymer ratio also had a significant effect on sol fraction. With an increase in PEG-DA concentration more acrylate double bonds were available for crosslinking. Thus, more crosslinks may be formed resulting in lower sol fraction. However, in the case of mPEG block length of the P(PF-co-EG), which was a dangling
chain and did not participate in the network structure, no significant effect was seen on sol fraction. The swelling due to the increased mPEG block length was independent of the degree of crosslinking. The hydrogel may be entirely crosslinked, but the increased PEG block lengths will allow more water to diffuse into the hydrogel matrix.

The second property of the hydrogel studied was the onset of gelation. The large effect due to mPEG block length could be due to the change in the viscosity of the hydrogel formulation, with hydrogels synthesized from copolymers containing mPEG 5000 being more viscous than those synthesized from mPEG 750. For example, the magnitude of the pre-crosslinked complex viscosity of formulation 4 (synthesized with P(PF-co-EG) of mPEG molecular weight 5000) and formulation 10 (synthesized with P(PF-co-EG) of mPEG molecular weight 750) was 2.9±0.2 and 7.0±0.2 Pa·s, respectively.

The APS concentration played a significant role in determination of the onset of gelation. An increase in APS concentration resulted in a decrease in the onset of gelation time, which could be due to increased free-radical production. For a particular formulation, varying the APS concentration was sufficient for a significant change in the gelation time. This provides a simple method of controlling the crosslinking time. The range of onset of gelation times were similar to those reported by Peter et al. who crosslinked the PPF homopolymer with NVP and used benzoyl peroxide as an initiator (Peter et al., 1999).

The third property characterized was the mechanical strength of the hydrogels. When the mPEG block length was increased, the tensile strength and modulus decreased. Due to the steric hindrance from the longer PEG chains, the molecular weight between
crosslinks was greater, reducing the tensile strength and modulus. On the other hand increasing the PEG-DA: P(PF-co-EG) ratio increased the tensile strength and modulus. More crosslinks may have formed due to the increased number of the reactive acrylate bonds from the PEG-DA ultimately resulting in greater crosslinking density. The addition of more crosslinks increased the tensile strength and modulus of the hydrogels.

The mechanical strength of the copolymer hydrogels was stronger when compared to other crosslinked PEG and poly(hydroxyethyl methacrylate) hydrogels (Iza et al., 1998; Jarvie et al., 1998; Mellott et al., 2001; Temenoff et al., 2002). Suggs et al. studied the mechanical strength of P(PF-co-EG) copolymer hydrogels using NVP as a crosslinking agent (Suggs et al., 1998). In this case, the copolymer was transesterified with PPF and difunctional PEG. The tensile strengths and moduli of the hydrogels in this study were generally lower when compared to the results in Suggs et al. This discrepancy may be because of the use of difunctional PEG which allows the formation of copolymers with multiple PPF blocks. The presence of multiple PPF blocks offers more fumarate double bond for crosslinking thereby increasing mechanical strength. The copolymers used in this study possess PPF with terminal PEG blocks, (Behravesh et al., 2002) with the potential to resist protein adsorption, the first step in the cell adhesion cascade. Moreover, a different initiator system and crosslinking agent was used in this study.

An important point to note was that the strength range of the hydrogels was comparable to that of the human blood vessel. Previous studies have shown that the tensile modulus of the human pulmonary artery is 2.7 Mpa (Carr-White et al., 2000). This study has shown that these hydrogels have tensile moduli in the range of 0.4 to 3.3 MPa.
These copolymer hydrogels may be suitable for use in cardiovascular applications. By changing all four parameters studied, the mechanical properties of the hydrogel may be manipulated according to the application for which it will be used. However, future studies in controlling the mechanical strength of these hydrogels should be focused on the PEG block length and PEG-DA: copolymer ratio.

In order to better quantify the effect of structure on the physical properties of the hydrogels, \( M_e \) values were approximated using the swelling and mechanical data. It is important to note that these values are only estimates since the hydrogel networks studied are not perfect, affecting the accuracy of Equation 3. Increasing the mPEG block length increased the \( M_e \) of the hydrogels. The steric hindrance of the larger PEG chains may have prevented efficient crosslinking from occurring resulting in larger \( M_e \)'s. In contrast, increasing the PEG-DA:P(PF-co-EG) ratio decreased the \( M_e \) of the hydrogels. The increased presence of the lower molecular weight PEG-DA may result in a more tightly bound hydrogel network as noted in the swelling studies. Since there was an increase in the number of short crosslinks formed, the overall \( M_e \) was also decreased. This result was significant because it shows that the \( M_e \) or mesh size of the copolymer hydrogel can be controlled by merely changing the concentration of crosslinking agent. It is noteworthy that the largest \( M_e \) was found in the formulation with the low PEG-DA:P(PF-co-EG) ratio and the high mPEG block length. In comparison, the formulation with the low mPEG block length and the high PEG-DA:P(PF-co-EG) ratio had the smallest \( M_e \). Upon further inspection of the swelling data, these formulations also had the greatest and least equilibrium water content of all the formulations, corroborating their respective \( M_e \) values.
The final property examined was the endothelial cell adhesion to the hydrogels. The results showed that mPEG block length had the greatest effect on endothelial cell adhesion. This effect could be explained due to the possible increase in hydrophobicity of the hydrogels with the smaller mPEG block lengths. This increased hydrophobicity allows more proteins to adsorb to the hydrogel surface and thus more cells to attach (Tziampazis et al., 2000). Additionally, the greater Mₐ in the hydrogels with the high mPEG block lengths may allow the long mPEG chains to freely extend to the surface. Thus, the steric repulsion from these long dangling PEG chains may repel the adsorption of proteins and therefore the adhesion of cells (Jeon et al., 1991). These results are typical of PEG containing hydrogels. Drumheller et al. have shown decreasing fibroblast cell adhesion with increasing PEG block length (Drumheller and Hubbell, 1995). Other studies have revealed decreasing cell adhesion with increasing PEG surface concentration (Tziampazis et al., 2000).

Increased cell adhesion was also seen on hydrogels with the higher PEG-DA:copolymer ratio. With increased PEG-DA concentration, the swelling of the hydrogels was decreased. As noted before, increasing the PEG-DA concentration decreases the Mₐ of the hydrogels. Less water may be able to diffuse into the more highly crosslinked hydrogel inhibiting the mobility of and reducing the surface concentration of the dangling PEG chains, thereby promoting protein adsorption. Although all parameters studied had significant effects on endothelial cell adhesion, mPEG block length of the copolymer, by far, had the greatest effect. Future efforts to influence cell adhesion should focus primarily on varying the dangling mPEG block length of the copolymer.
These P(PF-co-EG) hydrogels provide the necessary mechanical properties for use in cardiovascular application and show potential for use as macroporous scaffolds for tissue engineering (Behravesh et al., 2002). The overall resistance to cell adhesion provided by these copolymer hydrogels is attractive for tissue engineering applications where the prevention of unwanted adhesion of cells is required. The addition of covalently linked peptides targeting specific cell populations may provide an ideal biomaterial as a cell specific substrate for these applications.

4.5 Summary

Different P(PF-co-EG) hydrogels were synthesized by varying the mPEG block length, APS concentration, AA concentration, and PEG-DA:P(PF-co-EG) weight ratio. These hydrogels were characterized by examining the equilibrium water content, sol fraction, onset of gelation, mechanical strength, and endothelial cell adhesion. This study showed that properties of the hydrogel can be tailored by varying certain factors involved with the fabrication and the structure of the hydrogel. More importantly, by varying either the mPEG block length or the PEG-DA: P(PF-co-EG) weight ratio, the molecular weight between crosslinks can be significantly changed to manipulate the mechanical properties of and cell adhesion to the hydrogel. The results of this study showed that an injectable P(PF-co-EG) based hydrogel utilizing a water soluble crosslinking system may be useful for tissue engineering applications.
Chapter 5

Cell Adhesion to Bulk RGD Modified Poly(Propylene Fumarate-co-Ethylene Glycol) Hydrogels

5.1 Introduction

In the previous chapter, several properties of the copolymer hydrogel were characterized. One of the goals of the overall project was to use the material to promote the regeneration of the endothelium after angioplasty. A means to accomplish this would be to incorporate endothelial cell specific ligands into the hydrogel to promote the adhesion and migration of endothelial cells from the periphery of the angioplasty site. The first step towards this goal is test the feasibility of bulk modification of the copolymer hydrogel. In this study, the model cell adhesion peptide sequence, RGD, was covalently incorporated into the bulk hydrogel network. To assess the functionality of the peptide, the cell adhesion of three different cell types was tested.

5.2 Experimental

5.2.1 Materials

Diethyl fumarate (Acros, Pittsburgh, PA), propylene glycol (Acros), and monomethyl ether poly(ethylene glycol) of nominal molecular weights of 750 and 5000 (Aldrich, Milwaukee, WI) were used as received. Ascorbic acid (AA) (Sigma, St. Louis, MO) and ammonium persulfate (APS) (Acros) were used as received. All solvents were purchased from Aldrich as reagent grade. Hydroquinone (Aldrich) and zinc chloride (Aldrich) were also used as received. Poly(ethylene glycol) diacrylate of nominal molecular weight 700 was purchased from Aldrich.
5.2.2 Polymer Synthesis

PPF was synthesized in a two step reaction as previously described (Shung et al., 2002). Briefly, diethyl fumarate and propylene glycol were added to a three-necked round bottomed flask in a molar ratio of 1:3 respectively. In addition, hydroquinone was added as a crosslinking inhibitor and zinc chloride was added as a catalyst, in a 0.01:0.002:1 molar ratio to diethyl fumarate, respectively. The solution was submerged in an oil bath and mechanically mixed using an overhead stirrer under a nitrogen blanket at a maximum temperature of 150° C. Ethanol, a byproduct of the reaction, was collected as a distillate. After removal of the ethanol, the solution was allowed to cool to 100° C and then placed under vacuum (<1 mmHg). During the transesterification, propylene glycol was driven off and collected as a distillate.

The block copolymer P(PF-co-EG) was synthesized as previously described (Behravesh et al., 2002). Monomethoxy poly(ethylene glycol) (mPEG) of nominal molecular weight 2000 was added to the flask in a 1:2 PPF:mPEG molar ratio. The transesterification reaction for the copolymerization was carried out at a vacuum of less than 1 mmHg, with a maximum reaction temperature of 160° C. The extent of the reaction was monitored using gel permeation chromatography (GPC) by checking for the reduction of the free mPEG peak.

To purify the polymer, the resulting product was dissolved in dichloromethane, filtered through a Buchner funnel, and precipitated into ethyl ether under mechanical stirring. Zinc chloride and hydroquinone were soluble in ethyl ether and thus, were removed in the precipitation process. The powdery copolymer was isolated by filtration
through a Buchner funnel and subsequently dried under vacuum at room temperature to remove residual organic solvent.

5.2.3 Peptide PEG Acrylate Synthesis

Peptides were covalently attached to an acrylated PEG chain spacer using a procedure adopted from Hern and Hubbell (Hern and Hubbell, 1998). The peptide was first dissolved in a 50 mM sodium bicarbonate buffer, pH 8.2, at a concentration of 1 mg/mL. The acryloyl-PEG-N-hydroxysuccinimide ester (Acr-PEG-NHS) (MW 3400) (Shearwater Polymer, Birmingham, Alabama) was dissolved separately in 50 mM sodium bicarbonate buffer, pH 8.2. The Acr-PEG-NHS was reacted to the peptide in a 2:1 molar ratio for 2 h, respectively. The solution was dialyzed for 48 h to removed free peptide and any reaction by products (Spectrum, MWCO 2000). Afterwards the reaction solution was frozen and lyophilized for 24 h.

5.2.4 Bulk Peptide Modified Hydrogel Fabrication

The copolymer which had a PPF molecular weight 1500 and mPEG of molecular weight 2000 was first dissolved in double distilled water (ddH₂O) with an initial water content of 50 wt%. PEG-DA of nominal molecular weight 700 was added as a crosslinking agent in a weight ratio of either 1:2 with respect to copolymer. The acrylated PEG peptide was added to the polymer solution at a concentration of 1 μmol/mL (Figure 5-1). The prepolymer solution was centrifuged to remove air bubbles. APS was then added to the prepolymer at a concentration of 0.01 M, mixed, and centrifuged. Finally,
Figure 5-1. Fabrication of peptide modified P(PF-co-EG)/PEG-DA networks
the ascorbic acid (AA) was added to the mixture at a concentration of 0.01 M and cast in between two glass plates. The copolymer solution was left overnight at 37°C to crosslink. The resulting hydrogel was swollen in phosphate buffered saline (PBS) (GibcoBRL, Grand Island, NY) for 24 hours to reach equilibrium. The peptide modified hydrogels contained either GRGDS (Bachem) or GRDGS (Bachem) peptide. Two glass plates. The copolymer solution was left overnight at 37°C to crosslink (Figure 5-1). The resulting hydrogel was swollen in phosphate buffered saline (PBS) (GibcoBRL, Grand Island, NY) for 24 hours to reach equilibrium. The peptide modified hydrogels contained either GRGDS (Bachem) or GRDGS (Bachem) peptide.

5.2.5 Cell Culture

Human umbilical vein endothelial cells, human vascular smooth muscle cells and human dermal fibroblasts were obtained from American Tissue Culture Company (ATCC, Manassas, VA). All cell types were in maintained in Dulbecco’s Modified Eagle Medium (Sigma) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Product, Calabasas, CA) at 37°C. Cells of passage 33 were used for the cell adhesion experiment.

5.2.6 Cell Adhesion

The cells were rinsed with PBS and then trypsinized for 10 minutes. The cells were then resuspended in 10 mL of medium and centrifuged at 1500 rpm. After aspirating the medium, the cells were resuspended at a concentration of 56,400 cells/mL.
The hydrogel films were cut into 21 mm diameter disks using a cork borer. The films were dipped in 70% ethanol and then soaked in sterile PBS for 24 hours under exposure to UV light. The disks were placed at the bottom of 12 well tissue culture plates. Annular stainless steel rings with inner diameter of 15.5 mm were used to hold the films in place. The stainless steel rings were sterilized by autoclave prior to the experiment. The cells were seeded onto the hydrogels by pipetting 1 mL of the cell suspension into the wells for a seeding density of 30,000 cells/cm². After 8 hours, the wells were rinsed with PBS and the cells were trypsinized and counted using a Coulter Multisizer 3 (Beckman Coulter, Fullerton, CA).

5.2.7 Statistics

Statistical analysis was performed using analysis of variance (ANOVA) with a 95% confidence level (p<0.05). Statistically significant differences among experimental groups were determined using a one-way Tukey's HSD (highly statistically different) analysis.

5.3 Results and Discussion

The cell adhesion of three different cell lines on to bulk peptide modified hydrogels was measured 8 h after cell seeding. Two different peptide sequences were incorporated into the hydrogel, the ubiquitous GRGDS sequence and a scrambled sequence, GRDGS. The controls tested were tissue culture poly(styrene) (TCPS) and unmodified hydrogel. The results are shown in figures 5-2,5-3, and 5-4. For all three cell types, significantly lower cell adhesion was seen on non-peptide modified hydrogels
Figure 5-2. Adhesion of endothelial cells. Error bars represent means ± standard deviation for n=3. The symbol (*) indicates statistical significance from the non-peptide modified hydrogel. The (**) symbol indicates statistical significance from TCPS controls.
Figure 5-3. Adhesion of smooth muscle cells. Error bars represent means ± standard deviation for n=3. The symbol (*) indicates statistical significance from the non-peptide modified hydrogel. The (**) symbol indicates statistical significance from TCPS controls.
Figure 5-4. Adhesion of fibroblasts. Error bars represent means ± standard deviation for n=3. The symbol (*) indicates statistical significance from the non-peptide modified hydrogel. The (**) symbol indicates statistical significance from TCPS controls.
compared to TCPS controls. This result was to be expected. It is well known that protein adsorption is one of the first steps in the cell adhesion cascade. In the case of the copolymer hydrogel without modification, the long, dangling PEG chains may repel the surface adsorption of proteins and prevent cell adhesion (Jeon et al., 1991). The previous chapter showed that cell adhesion was decreased when the PEG block length was increased. Studies have also been done showing decreased cell adhesion with increasing PEG content (Tziampazis et al., 2000).

In comparison, the GRGDS modified hydrogels showed significant cell adhesion for all cell types when compared to non-peptide modified controls. The cell adhesion for all cell types was not statistically different than to TCPS controls. To ensure that the GRGDS peptide sequence was present on the hydrogel surface, the PEG spacer length used was 3400 as compared to a PEG block length of 2000 in the copolymer. Previous studies have shown that PEG spacer length is critical in ensuring that the peptide is presented at the hydrogel surface (Behravesh et al., 2002; Shin et al., 2002). The RGD sequence has been shown to be a potent mediator of cell adhesion for many different cell types (Hirano et al., 1993). Thus, it was not unexpected that all three cell lines showed a marked increase in cell adhesion compared to the non-peptide modified hydrogel. The adhesion and migration of marrow stromal cells has also been studied on to GRGDS modified P(PF-co-EG) based hydrogels (Behravesh et al., 2002). Increasing the GRGDS concentration in the hydrogel network increased the number of well spread cells on to the functionalized hydrogel. In this study, only a concentration of 1 μmol/mL of GRGDS was investigated. When directly compared to the adhesion of marrow stromal cells on to hydrogel with the same concentration of GRGDS, the percentage of cells adhered in this
study was lower. However, in the marrow stromal study, cells were counted after 12 hours of culture compared to 8 hours of culture in the present study. During the 4 hour difference, some of the marrow stromal cells may have proliferated and slightly increased the cell numbers. In general, the adhesion of the cells between both studies were comparable.

Studies have shown that cells adhere non-specifically to surfaces due to charge interactions (Webb et al., 1998; Ohgaki et al., 2001). To make sure that the cells were attaching through a specific integrin-peptide interaction and not due to any charged functional groups contained in the peptide, a scrambled peptide sequence, GRDGS was incorporated into the hydrogels as well. For all cell types, minimal cell adhesion was observed compared to GRGDS modified hydrogels and TCPS controls. The cell adhesion on to the GRDGS for all cell types was not significantly different than on to unmodified hydrogels. This result showed that the cells were adhering due to specific recognition of the GRGDS sequence.

5.4. Summary

Copolymer hydrogel networks modified with the GRGDS peptide sequence were fabricated. The adhesion of endothelial cells, smooth muscle cells, and fibroblasts was tested to assess the bioactivity of the incorporated peptides. In all cases, the hydrogels modified with GRGDS showed significantly increased cell adhesion when compared to unmodified controls. Furthermore, cells did not attach to hydrogels modified with the GRDGS scrambled sequence discounting any cell adhesion due to positive charges. These results show that the copolymer hydrogel can be modified with bioactive peptides for the promotion of cell adhesion. Such a bioactive hydrogel may be useful for the
regeneration of the endothelium after balloon angioplasty. Further work must be done to test the feasibility of modifying the network with cell specific peptide sequences.
Chapter 6 - Conclusions

We have developed an injectable hydrogel utilizing P(PF-co-EG) and a water soluble crosslinking system that may be useful for cardiovascular applications. The use of a water soluble crosslinking system is advantageous because its precludes the use of toxic organic solvents. Several properties of the hydrogel were characterized including equilibrium water content, sol fraction, onset of gelation, mechanical strength, cell adhesion, and molecular weight between crosslinks. In addition, the kinetics of the PPF reaction using zinc chloride as a catalyst, an integral part of the copolymer synthesis, were characterized.

We have shown that the rate of the PPF reaction was dependent on the transesterification temperature. The initial rate constants were quantified and shown to be dependent on temperature. We also have shown that at a reaction temperature above 200°C, gelation of the PPF occurs. The synthesized diester intermediate and PPF were characterized spectrometrically using MS, NMR, and FT-IR. The mass spectrogram showed that the intermediate indeed is mostly diester along with excess propylene glycol.

Several parameters involved in the fabrication of the hydrogel were varied to characterize the different properties. These parameters included the mPEG block length, the AA concentration, the APS concentration, and the PEG-DA:copolymer ratio. Our study showed that the material properties could be modulated by varying some of these parameters. For example, the onset of gelation time was shown to be reduced by increasing the APS concentration. The onset of gelation is critical in clinical applications since it defines the amount of time the physicians will have to work with the material
before it crosslinks. The copolymer hydrogel was also shown to have sufficient mechanical strength for cardiovascular applications. These copolymer hydrogels were shown to be mechanically stronger compared to other PEG based hydrogels. Another property that could be modulated was the molecular weight between crosslinks. Our study showed that the $M_c$ could be manipulated merely by changing the concentration of the crosslinking monomer. This quality is important for future use in drug delivery applications where the diffusion of large molecules is required.

We also have shown that these copolymer hydrogels can be functionalized with bioactive peptides to promote cell adhesion to an otherwise non-cell adhesive surface. If the hydrogel were modified with endothelial cell specific peptides, in theory, it could promote the migration and adhesion of endothelial cells from the periphery of the balloon angioplasty site. However, the feasibility of such a system remains to be seen. Although, much more work needs to be done, we feel that the copolymer hydrogel in conjunction with a water soluble crosslinking system is certainly a suitable candidate for use in cardiovascular applications as well as other tissue engineering applications.
References


