RICE UNIVERSITY

Synthesis of an Injectable Biodegradable Biomimetic Macroporous Hydrogel Scaffold for Bone Tissue Engineering

By

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ABSTRACT

Synthesis of an Injectable Biodegradable Biomimetic Macroporous Hydrogel

Scaffold for Bone Tissue Engineering

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The objective of this work was to direct cell-biomaterial interaction by utilizing materials that reduce non-specific cell adhesion and covalently modify them with a cell adhesive peptide sequence to promote cell adhesion by a specific receptor-ligand interaction. The potential of poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) based hydrogels as biomimetic materials for controlling osteoblastic cell interaction and modulating cell response was examined.

First, the block copolymer P(PF-co-EG) was synthesized by transesterification of the homopolymers poly(propylene fumarate) and methoxy poly(ethylene glycol) to result in a block copolymer with terminal poly(ethylene glycol) (PEG) blocks. The effect of the PEG block length on the thermoreversible properties of the copolymer in water was examined. Next, P(PF-co-EG)-based hydrogels were crosslinked to thin films. Increasing the PEG block length of P(PF-co-EG) reduced marrow stromal osteoblast adhesion. Hydrogels were bulk-modified with an RGDS ligand attached to the backbone of the hydrogel with a PEG spacer arm. Marrow stromal osteoblast adhesion and
migration was found to be dependent on the bulk concentration of RGDS peptide. In addition, the availability of the RGDS peptide at the surface of the bulk-modified hydrogels was dependent on the ratio of the PEG block length of P(PF-co-EG) and the PEG spacer arm linking the peptide to the hydrogel. A model biotin ligand was utilized to quantify the surface concentration of biotin bulk-modified hydrogels using an enzyme linked immunosorbent assay.

Macroporous hydrogels based on P(PF-co-EG) were synthesized by the same free-radical crosslinking approach as the thin film with potential for in situ formation. The effects of the free-radical initiation system and the carbon dioxide porogen precursor on the extent of crosslinking and morphology of the resulting macroporous hydrogel were examined. Moreover, these macroporous hydrogels were found to degrade hydrolytically in a bulk fashion with a decrease in material properties and dry weight, and no significant difference in porosity of the hydrogels. Degradation was dependent on the crosslinking density of the hydrogel. Finally, marrow stromal cells showed markers typical of differentiation to the osteoblastic phenotype when cultured for 28 days under conditions to promote their osteoblastic phenotype on RGDS-modified biomimetic macroporous hydrogels.
ACKNOWLEDGEMENTS

Success is not final; failure is not fatal; it is the courage to continue that counts.
Sir Winston Churchill

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# TABLE OF CONTENTS

Abstract................................................................................................................................. ii
Acknowledgements............................................................................................................... iv
Table of Contents.................................................................................................................. v
List of Figures......................................................................................................................... vii
List of Tables........................................................................................................................ ix
List of Abbreviations........................................................................................................... x

Chapter 1  Introduction ........................................................................................................... 1

Chapter 2  Synthetic Biodegradable polymers for orthopaedic applications ................. 3
  2.1 Introduction ................................................................................................................... 3
  2.2 Poly(α-hydroxy esters) ................................................................................................. 6
  2.3 Poly(phosphazenes) ..................................................................................................... 9
  2.4 Poly(anhydrides) ........................................................................................................ 11
  2.5 Poly(propylene fumarate) ......................................................................................... 16
  2.6 Summary ...................................................................................................................... 22

Chapter 3  Objectives ........................................................................................................... 24

Chapter 4  Synthesis and characterization of Triblock copolymers of Methoxy Poly(ethylene glycol) and Poly(propylene fumarate) ....................... 26
  4.1 Introduction ................................................................................................................... 27
  4.2 Material and Methods ................................................................................................. 29
  4.3 Results and Discussion ............................................................................................... 34
  4.4 Conclusions .................................................................................................................. 45

Chapter 5  Adhesion and Migration of Marrow-Derived Osteoblasts on Injectable In Situ Crosslinkable Poly(propylene fumarate-co-ethylene glycol) Based Hydrogels with a Covalently Linked RGDS Peptide ............................................ 46
  5.1 Introduction ................................................................................................................... 47
  5.2 Materials and Methods ............................................................................................... 50
  5.3 Results ........................................................................................................................ 57
  5.4 Discussion .................................................................................................................... 68
  5.5 Conclusions .................................................................................................................. 73

Chapter 6  Quantification of the surface concentration of bulk biotin-modified hydrogels ......................................................................................................................... 74
  6.1 Introduction ................................................................................................................... 75
  6.2 Materials and Methods ............................................................................................... 77
  6.3 Results ........................................................................................................................ 87
  6.4 Discussion .................................................................................................................... 93
  6.5 Conclusions .................................................................................................................. 97
Chapter 7  Synthesis of In Situ Crosslinkable Biodegradable Macroporous Hydrogels Based on Poly(propylene fumarate-co-ethylene glycol)...... 99
  7.1 Introduction .............................................................................. 100
  7.2 Materials and Methods............................................................. 103
  7.3 Results..................................................................................... 110
  7.4 Discussion............................................................................... 121
  7.5 Conclusions........................................................................... 125

Chapter 8  Evaluation of the In Vitro Degradation of Macroporous Hydrogels Using Gravimetry, Confined Compression Testing, and Microcomputed Tomography............................................. 127
  8.1 Introduction............................................................................. 128
  8.2 Materials and Methods............................................................. 130
  8.3 Results..................................................................................... 138
  8.4 Discussion............................................................................... 149
  8.5 Conclusions........................................................................... 153

Chapter 9  The osteogenic potential of marrow-stromal cells cultured on macroporous hydrogels .......................................................... 154
  9.1 Introduction............................................................................. 155
  9.2 Materials and Methods............................................................. 157
  9.3 Results..................................................................................... 164
  9.4 Discussion............................................................................... 171
  9.5 Conclusions........................................................................... 174

Bibliography .................................................................................. 176
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Structure of poly(α-hydroxy esters)</td>
<td>6</td>
</tr>
<tr>
<td>2-2</td>
<td>Structure of poly(phosphazenes)</td>
<td>9</td>
</tr>
<tr>
<td>2-3</td>
<td>Structure of poly(anhydrides)</td>
<td>12</td>
</tr>
<tr>
<td>2-4</td>
<td>Diacids used in synthesis of poly(anhydrides)</td>
<td>13</td>
</tr>
<tr>
<td>2-5</td>
<td>Structure of several imides for synthesis of poly(anhydride-co-imides)</td>
<td>13</td>
</tr>
<tr>
<td>2-6</td>
<td>Degradation profiles for poly(anhydride-co-imides)</td>
<td>15</td>
</tr>
<tr>
<td>2-7</td>
<td>Structure of methacrylated sebacic acid</td>
<td>16</td>
</tr>
<tr>
<td>2-8</td>
<td>Structure poly(propylene fumarate)</td>
<td>17</td>
</tr>
<tr>
<td>2-9</td>
<td>Mechanical properties of PPF samples degraded in vitro</td>
<td>20</td>
</tr>
<tr>
<td>2-10</td>
<td>Mechanical properties of PPF samples degraded in vivo</td>
<td>21</td>
</tr>
<tr>
<td>4-1</td>
<td>Synthesis of P(PF-co-EG)</td>
<td>31</td>
</tr>
<tr>
<td>4-2</td>
<td>Gel permeation chromatographs of P(PF-co-EG)</td>
<td>36</td>
</tr>
<tr>
<td>4-3</td>
<td>Proton NMR spectrum P(PF-co-EG)</td>
<td>40</td>
</tr>
<tr>
<td>4-4</td>
<td>FTIR spectra of P(PF-co-EG)</td>
<td>41</td>
</tr>
<tr>
<td>4-5</td>
<td>Sol-gel transition temperature of P(PF-co-EG)</td>
<td>44</td>
</tr>
<tr>
<td>5-1</td>
<td>Digitized images of marrow-stromal osteoblasts adhesion on P(PF-co-EG) hydrogels</td>
<td>60</td>
</tr>
<tr>
<td>5-2</td>
<td>Marrow-derived osteoblast adhesion to P(PF-co-EG) hydrogels</td>
<td>61</td>
</tr>
<tr>
<td>5-3</td>
<td>Marrow-derived osteoblast adhesion to P(PF-co-EG) hydrogels with soluble RGD peptide</td>
<td>62</td>
</tr>
<tr>
<td>5-4</td>
<td>Distribution of surface area covered by marrow-stromal osteoblasts</td>
<td>63</td>
</tr>
<tr>
<td>5-5</td>
<td>Digitized images of marrow-stromal osteoblast migration</td>
<td>65</td>
</tr>
<tr>
<td>5-6</td>
<td>Surface area of migrating marrow-stromal osteoblasts</td>
<td>66</td>
</tr>
<tr>
<td>5-7</td>
<td>Proliferation of marrow-derived osteoblasts</td>
<td>67</td>
</tr>
<tr>
<td>6-1</td>
<td>Water soluble biotin molecule</td>
<td>79</td>
</tr>
<tr>
<td>6-2</td>
<td>Structure of biotin-modified crosslinked hydrogel</td>
<td>80</td>
</tr>
<tr>
<td>6-3</td>
<td>Characterization of the hydrogel</td>
<td>89</td>
</tr>
<tr>
<td>6-4</td>
<td>Representative luminescence images</td>
<td>91</td>
</tr>
<tr>
<td>6-5</td>
<td>Surface concentration of bulk modified hydrogels</td>
<td>92</td>
</tr>
<tr>
<td>7-1</td>
<td>Stereological evaluation of macroporous hydrogels</td>
<td>109</td>
</tr>
<tr>
<td>7-2</td>
<td>Main effects on sol fraction of P(PF-co-EG) hydrogels</td>
<td>112</td>
</tr>
<tr>
<td>7-3</td>
<td>Main effect on porosity of P(PF-co-EG) hydrogels</td>
<td>113</td>
</tr>
<tr>
<td>7-4</td>
<td>Main effect on surface area density of P(PF-co-EG) hydrogels</td>
<td>114</td>
</tr>
<tr>
<td>7-5</td>
<td>Variation of sol fraction of P(PF-co-EG) hydrogels</td>
<td>116</td>
</tr>
<tr>
<td>7-6</td>
<td>Variation of porosity of P(PF-co-EG) hydrogels</td>
<td>117</td>
</tr>
<tr>
<td>7-7</td>
<td>Variation of surface area density of P(PF-co-EG) hydrogels</td>
<td>118</td>
</tr>
<tr>
<td>7-8</td>
<td>Representative SEM of macroporous hydrogels</td>
<td>119</td>
</tr>
<tr>
<td>7-9</td>
<td>Probability distribution of pore sizes</td>
<td>120</td>
</tr>
<tr>
<td>7-10</td>
<td>Assessment of profile size distribution</td>
<td>125</td>
</tr>
<tr>
<td>8-1</td>
<td>Illustration of the morphological parameters pore wall thickness and pore edge length</td>
<td>136</td>
</tr>
<tr>
<td>8-2</td>
<td>Weight loss and total water content of macroporous hydrogels</td>
<td>140</td>
</tr>
<tr>
<td>8-3</td>
<td>Representative stress/strain curves of macroporous hydrogels</td>
<td>141</td>
</tr>
<tr>
<td>8-4</td>
<td>Confined compressive modulus of macroporous hydrogels</td>
<td>142</td>
</tr>
<tr>
<td>8-5</td>
<td>Determination of threshold level and height of sampled cylinder</td>
<td>143</td>
</tr>
<tr>
<td>8-6</td>
<td>Representative μCT scans before and after 12 weeks of degradation</td>
<td>145</td>
</tr>
<tr>
<td>8-7</td>
<td>Morphology of macroporous hydrogels</td>
<td>146</td>
</tr>
<tr>
<td>8-8</td>
<td>Hydrogel equilibrium water content of macroporous hydrogels</td>
<td>148</td>
</tr>
</tbody>
</table>

<p>| 9-1 | Cellularity of macroporous hydrogel scaffolds | 166 |
| 9-2 | Alkaline phosphatase activity of macroporous hydrogel scaffolds | 167 |
| 9-3 | Calcium content of macroporous hydrogel scaffolds | 169 |
| 9-4 | Representative confocal depth-projection micrographs | 170 |</p>
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Mechanical properties of bone</td>
<td>5</td>
</tr>
<tr>
<td>4-1</td>
<td>Molecular weight determined by GPC relative to polystyrene standards</td>
<td>35</td>
</tr>
<tr>
<td>4-2</td>
<td>Molecular weight determined by proton NMR</td>
<td>39</td>
</tr>
<tr>
<td>4-3</td>
<td>Lower critical solution temperature of P(PF-co-EG)</td>
<td>42</td>
</tr>
<tr>
<td>5-1</td>
<td>Characterization of RGD-modified hydrogels</td>
<td>58</td>
</tr>
<tr>
<td>6-1</td>
<td>Biotin modified hydrogel characterization</td>
<td>87</td>
</tr>
<tr>
<td>6-2</td>
<td>Bulk concentration of biotin modified hydrogels</td>
<td>88</td>
</tr>
<tr>
<td>6-3</td>
<td>Surface coverage of IgG on hydrogels</td>
<td>92</td>
</tr>
<tr>
<td>7-1</td>
<td>Macroporous hydrogel synthesis: two-level factorial design</td>
<td>105</td>
</tr>
<tr>
<td>7-2</td>
<td>High and low measured values of factorial experiment</td>
<td>111</td>
</tr>
<tr>
<td>8-1</td>
<td>Macroporous hydrogel degradation: two-level factorial design</td>
<td>133</td>
</tr>
<tr>
<td>9-1</td>
<td><em>In vitro</em> culture: description of experimental groups</td>
<td>159</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

| 2-D | Two-dimensional          |
| 3-D | Three-dimensional        |
| AH  | L-Ascorbic acid          |
| ALPase | Alkaline phosphatase    |
| ANOVA | Analysis of variance   |
| APS | Ammonium persulfate      |
| ATR | Attenuated total reflectance |
| BP  | Benzoyl peroxide         |
| DDW | Distilled deionized water|
| DMEM | Dulbecco’s modified eagle medium |
| DMT | N,N-dimethyl-p-toluidine |
| DNA | Deoxyribonucleic acid    |
| FBS | Fetal bovine serum       |
| FTIR | Fourier transform infrared |
| GPC | Gel permeation chromatography |
| HA  | Hydroxyapatite           |
| HCl | Hydrochloric acid        |
| HSD | Highly statistically different |
| MMA | Methyl methacrylate      |
| Mn  | Number average molecular weight |
| mPEG | Methoxy poly(ethylene glycol) |
| MPH | Macroporous hydrogel     |
| MW  | Molecular weight         |
| NMR | Nuclear magnetic resonance |
| PBS | Phosphate buffered saline|
| PEG | Poly(ethylene glycol)    |
| PI  | Polydispersity index     |
| PPF | Poly(propylene fumarate) |
| P(PF-co-EG) | Poly(propylene fumarate-co-ethylene glycol) |
| PGA | Poly(glycolic acid)      |
| PLA | Poly(lactic acid)        |
| PLAL | poly(lactic acid-co-lysine) |
| PLGA | Poly(lactic-co-glycolic acid) |
| PMMA | Poly(methyl methacrylate) |
| RDG | Arg-Asp-Gly              |
| RGD | Arg-Gly-Asp              |
| SA  | Sebacic acid             |
| SB  | Sodium bicarbonate       |
| SD  | Standard deviation       |
| SEM | Scanning electron microscopy |
| β-TCP | Beta-tricalcium phosphate |
| TCPS | Tissue culture poly(styrene) |
| VP  | N-vinyl pyrrolidinone    |
CHAPTER 1

Introduction

The knowledge of interaction mechanisms between cells and biomaterials is significant for the performance of an implanted material as well as for the development of more effective therapies. Anchorage-dependent cell adhesion to substrates is the first step in the process of cell-surface interaction and affects subsequent cellular and tissue responses including migration, proliferation, and differentiation.

In orthopaedic and maxillo-facial fields, the early interaction between osteoblastic cells and their substrates affects the later mineralization process and determines the fate of the implant. In fact, critical to the success of implants is the development of a stable direct connection between osteoblastic cells and the surface of the implant.

Tissue engineering strategies for the regeneration of damaged orthopaedic and maxillo-facial tissues usually involve the use of a highly porous scaffold to serve as a substrate for the adhesion, migration, proliferation, and differentiation of osteoblasts. Synthetic bioresorbable polymers have been utilized as biomaterials for these scaffolds as will be discussed in the following chapter. These scaffolds have been used in a variety of approaches: to induce tissue ingrowth from surrounding areas, as a vehicle for cell transplantation, and as a vehicle for the delivery of bioactive molecules.

This work is focused on injectable scaffold based therapies for non-load bearing bone tissue engineering. Historically, a scaffold by itself is at best tissue conductive and does not induce tissue formation. The scaffold based tissue induction approach relies on the
availability and migration of osteoprogenitor cells into the scaffold guiding the growth and regeneration of the new bone tissue. Of critical importance to this approach is the interaction between the progenitor cell and the biomaterial, which is mediated on what the cell comes into contact with on the material. For many biomaterials, the proteins adsorbed on the surfaces of the material mediate this interaction. The objective of this work is to direct this cell-biomaterial interaction by utilizing materials that initially minimize non-specific cell adhesion and covalently modifying them with peptide sequences that have been shown to promote cell adhesion to allow for a specific and desired interaction between a cell and the biomaterial.

For therapies where in situ formation or injectability is required, the material needs to be modified in the bulk with the peptide segments given that surface modification after in situ formation is not possible. The availability of the peptide at the surface of the bulk-modified material to the cell is dependent on the physical and chemical properties and structure of the material. Therefore, the material used as a basis for these studies is of the utmost importance. The following chapter introduces the most promising candidates for this purpose.
CHAPTER 2

Synthetic Biodegradable Polymers for Orthopaedic Applications

Chapter Abstract

Synthetic biodegradable polymers offer an alternative to the use of autografts, allografts, and non-degradable materials for bone replacement. They can be synthesized with tailored mechanical and degradative properties. They can also be processed to porous scaffolds with desired pore morphologies conducive to tissue ingrowth. Moreover, they can be functionalized to induce tissue ingrowth and guide tissue regeneration. This review focuses on four classes of polymers which hold promise for orthopaedic applications: poly(α-hydroxy esters), poly(phosphazenes), poly(anhydrides), and poly(propylene fumarate) crosslinked networks.

2.1 Introduction

There is a continued attempt to develop synthetic degradable materials for use in orthopaedic and craniofacial surgery because of problems of supply and harvest site morbidity for autogenic bone, immunogenecity and potential disease transfer associated with allogenic bone, as well as stress shielding and particulate wear for non-degradable

materials such as poly(methyl methacrylate) (PMMA) [Bostrom et al., 1997]. Synthetic biodegradable polymers offer an ideal replacement material because of their 1) ease of synthesis, 2) unlimited supply, and 3) potential coupling of polymer degradation and removal with concurrent tissue regeneration. Polymers can be tailored with great flexibility to meet these criteria.

The most used degradable polymers for medical devices are poly (α-hydroxy esters) and poly(anhydrides). However, the use of these polymers in load-bearing orthopaedic applications for guided tissue growth is limited due to their poor mechanical properties compared to bone (Table 2-1) [Einhorn, 1996; Yaszemski et al., 1996a]. For trabecular bone, the strength and modulus are related to the density of the bone that varies greatly. Degradable materials must also maintain their mechanical integrity over a sufficient time period to allow the ingrowth of tissue necessary for bone formation [Uhrich et al., 1997].

One approach to bone replacement involves prefabricated polymer scaffolds for cell transplantation. These scaffolds present a large surface area for cell growth and reduce the diffusional barriers for material transport [Boyan et al., 1996]. To promote three dimensional tissue growth, nutrient diffusion and vascularization, highly porous scaffolds have been fabricated. Several methods have been proposed for fabrication of scaffolds of desired pore morphologies [Widmer et al., 1998].
Table 2-1. Mechanical properties of bone [Einhorn, 1996; Yaszemski et al., 1996b].

<table>
<thead>
<tr>
<th>Type of Bone</th>
<th>Apparent Density (Kg/m³)</th>
<th>Load</th>
<th>Strength (MPa)</th>
<th>Young’s Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical</td>
<td>1850</td>
<td>Longitudinal tension</td>
<td>78.8-151</td>
<td>17000-20000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Longitudinal compression</td>
<td>131-224</td>
<td>17000-20000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Longitudinal shear</td>
<td>53.1-70</td>
<td>3300</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transverse tension</td>
<td>51-56</td>
<td>6-13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transverse compression</td>
<td>106-133</td>
<td>6-13</td>
</tr>
<tr>
<td>Cancellous</td>
<td>100-1000</td>
<td>Compression</td>
<td>5-10</td>
<td>50-100</td>
</tr>
</tbody>
</table>

Another approach involves the use of *in situ* crosslinkable materials. The benefits of crosslinkable systems include high strength and ease of fashioning and flexibility during implantation. Acrylate and methacrylate moieties are the most common crosslinking functional groups. Important factors for *in situ* polymerization are the toxicity of the initiator system and the temperature rise during polymerization. An ideal synthetic substitute, irrespective of the approach, has to be osteoconductive and allow for neovascularization so cells from the surrounding tissue can migrate to and proliferate on the three-dimensional scaffold.

This paper reviews four potential synthetic biodegradable polymers including poly(ω-hydroxy esters), poly(phosphazenes), poly(anhydrides), and poly(propylene fumarate) crosslinked networks which exhibit favorable mechanical, physical, and chemical properties for use in load bearing orthopaedic applications.
2.2 Poly(α-hydroxy esters)

Poly(α-hydroxy esters) (Figure 2-1), specifically poly(lactic acid) (R=CH₃), poly(glycolic acid) (R=H), and their copolymers, have a long history of use as synthetic biodegradable materials. These polymers have been used as surgical sutures [Cutright et al., 1971], plates and screws for fracture fixation devices [Mayor et al., 1995], and artificial scaffolds for cell transplantation [Thomson et al., 1993]. Poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and their copolymers are biocompatible and are FDA approved for certain clinical uses [Mayor et al., 1995].

![Figure 2-1. Structure of poly(α-hydroxy esters)](image)

2.2.1 Synthesis

High molecular weight PLA and PGA are produced by ring opening polymerization of lactide and glycolide [Hollinger et al., 1995]. This ring opening polymerization can be accomplished in a molten state or in solution phase [Sawhney et al., 1998]. Self-reinforced polymers have been used in an attempt to increase the bending strength of the polymer. By using a sintering or drawing technique, polymers made of PGA can attain an elongation of 7 to 35 percent [Agrawal et al., 1995]. Ceramics have also been incorporated into PLA-PGA formulations for improved mechanical properties [Devin et al., 1996; Thomson et al., 1998]. Ceramics are structurally very similar to the mineralized
phase of bone. Calcium phosphate, in the form of synthetic hydroxyapatite and β-tricalcium phosphate (β-TCP), has been investigated both as a matrix by itself and as a reinforcing agent and has been shown to have osteoconductive potential. Poly(lactic-co-glycolic acid) (PLGA) copolymers have been reinforced with particulate hydroxyapatite (HA) [Devin et al., 1996]. A three-dimensional porous matrix of 85:15 PLGA and 47% porosity reinforced with short fibers of HA was shown to have a maximum compressive yield strength of 2.82 MPa with polymer to fiber weight ratios of 7:6 [Thomson et al., 1995].

2.2.2 Degradation

Most synthetic biodegradable polymers degrade by passive hydrolysis. Factors that influence their degradation rate include water uptake, pH and bond type [Suggs et al., 1996]. Crystallinity and steric hindrance also affect the degradation rate of the polymer. Poly(α-hydroxy acids) undergo non-enzymatic hydrolysis of the polyester bond resulting in either lactic or glycolic acid [Kopecek et al., 1983]. These polymers undergo bulk degradation leading to a sudden decrease of mechanical properties without a change in the overall dimensions of the polymer. Degradation of these polymers depends on the copolymer composition, molecular weight, crystallinity, thermal history, geometry and porosity [Agrawal et al., 1995]. Degradation times for various polymers and copolymers can range from weeks to years [Suggs et al., 1996].
2.2.3 Biocompatibility

Osteoblasts isolated from neonatal rat calvaria, cultured on PLGA films and three-dimensional porous scaffolds, has been shown to attach, proliferate, migrate, and show phenotypic expression of osteoblast markers, including increased ALPase activity and mineralized matrix formation [Ishaug et al., 1997; Ishaug et al., 1996]. Rat calvaria osteoblasts have also been cultured on PLGA/HA films and porous scaffolds with similar results of cell adhesion and proliferation when compared to PLGA scaffolds [Attawia et al., 1995b]. The morphology of osteoblast-like cells on these scaffolds is similar to tissue culture poly(styrene) (TCPS) and the cells express typical osteoblast markers [Attawia et al., 1995a].

2.2.4 Functional Poly(α-hydroxy esters)

Functionalization, by the conversion of end groups or addition of side chains with various groups including -OH, -NH2, -COOH, -COONa, and -CH=CH2, results in polymers that can be self crosslinked or crosslinked with proteins and other bioactive agents. Poly(lactic acid-co-lysine) copolymers [Barrera et al., 1995] with attached aspartic acid (PLAL-ASP) have been synthesized and crosslinked by UV polymerization [Elisseeff et al., 1997]. Covalent attachment of specific peptide sequences can allow interactions of polymer with cell membrane receptors [Bostrom et al., 1997]. To modulate mammalian cell adhesion an RGD peptide has been covalently attached to functionalized PLAL [Barrera et al., 1993]. This peptide can control the interactions of cells with the polymer scaffold [Cook, 1997]. For example, osteoblasts cultured under
specific conditions on glass modified with RGD peptides, when compared with scrambled peptide sequences, showed significant increase in mineralization [Dee et al., 1996].

2.3 Poly(phosphazenes)

Poly(phosphazenes) (PPHOS) are a unique class of polymers that contain alternating nitrogen and phosphorous with no carbon atoms in their backbone structure (Figure 2-2). The versatility of these polymers lies in the addition of side groups after the backbone synthesis.

\[ \text{N=P} \bigg[ \begin{array}{c} \text{R} \\ \text{R'} \end{array} \bigg]_n \]

**Figure 2-2. Structure of poly(phosphazenes)**

2.3.1 Synthesis

Poly(phosphazenes) are synthesized in a two step process. First a prepolymer, polydichlorophosphazene (R,R'Cl), is formed, most commonly through a ring-opening polymerization of the cyclic trimer in either the molten state or in solution phase [Allcock, 1998]. This reaction results in a polymer with a high weight average molecular weight of ~2,000,000, but also a relatively high polydispersity index. A living condensation polymerization of Me3SiN=P(O)Cl2 results in polydichlorophosphazene with a narrower molecular weight distribution, and allows for block and star copolymer formulations [Allcock, 1998]. The second step involves a macromolecular substitution of
the chlorine atoms with a desired nucleophile. Various organic nucleophiles can replace the chlorine atom to create polymers with a variety of physical and chemical properties [Wade et al., 1977]. This macromolecular substitution scheme also allows for the introduction of different types of side groups in the backbone of a particular polymer chain. Degradable PPHOS contain amino acid-ester side groups. Methylphenoxy substituted and either imidazolyl or ethyl glycinto substituted PPHOS have been synthesized for skeletal tissue regeneration [Laurencin et al., 1993]. PPHOS and PLGA have been mixed and fabricated using solvent casting techniques [Ibim et al., 1997]. These polymer blends have been shown to be miscible to one another.

2.3.2 Degradation

Degradation can be controlled by an increase in the amount of hydrolytically unstable side groups. With increasing incorporation of either imidazolyl or ethyl glycinate-substituted PPHOS, an increase in degradation rate is observed [Laurencin et al., 1993]. Also, for several formulations of PPHOS / PLGA blends, a near zero-order degradation rate was observed [Ibim et al., 1997].

2.3.3 Biocompatibility

Both poly[(imidazolyl) (methylphenoxy) phosphazene] and poly[(ethyl glycinate) (methylphenoxy) phosphazene] have been shown to support MC3T3-E1, osteoblast cell adhesion [Laurencin et al., 1993]. However, an increase in hydrophilicity of the imidazolyl substituted PPHOS lowered the attachment and growth of MC3T3-E1 cells. Three dimensional, porous matrices of poly(50% ethyl glycinate) (50% p-
methylphenoxy) phosphazene with pore size of 150-250 μm have been shown to be a good substrate for osteoblast-like cell attachment and growth [Laurencin et al., 1996].

2.3.4 Functional Poly(phosphazenes)

Schemes for functionalizing PPHOS do so after nucleophiles displace the chlorine atoms since addition of di-functional nucleophiles crosslinks the polymer. Functionalization of PPHOS has been accomplished both in bulk and at the surface [Allcock, 1998].

2.4 Poly(anhydrides)

Poly(anhydrides) (Figure 2-3), developed in part as drug delivery systems, have well-defined degradation characteristics [Leong et al., 1985] that can be controlled by varying the amount of hydrophobic or hydrophilic monomers such as 1,3-bis(p-carboxyphenoxy)propane (CPP) and sebamic acid (SA). Unlike polyesters, which degrade by a bulk degradation process leading to a sudden increase in acid production at the late stages of degradation, polyanhydrides degrade in a controlled fashion by surface erosion. Degradable anhydrides have been shown to be safe in two phase III clinical trials in drug delivery for brain tumor applications [Anseth et al., 1997; Laurencin et al., 1995].

The use of poly(anhydrides) in orthopaedic applications is limited due to their poor mechanical strength. Poly(anhydride-co-imides) were developed to meet some of the shortcomings of poly(anhydrides). By themselves, imides have good mechanical
properties due to their rigid units; however, they are not degradable. The imide component has been incorporated into the backbone of poly(anhydrides) to increase their mechanical strength for orthopaedic applications. In situ crosslinkable polymers have also been developed for orthopaedic use to allow the polymer to crosslink at the time of implantation and conform to irregularly shaped defects [Anseth et al., 1997].

![Figure 2-3. Structure of poly(anhydrides)](image)

2.4.1 Synthesis

Poly(anhydrides) are synthesized by the reaction of various diacids (Figure 2-4) with acetic anhydride under reflux conditions. Two imides used in the copolymerization with poly(anhydrides) are shown in Figure 2-5. The copolymer is synthesized by first activating the diacid with acidic anhydride and then adding the imide to give the imide-anhydride monomer [Uhrich et al., 1995]. The copolymer is synthesized by melt condensation polymerization of the imide-anhydride monomer and the desired anhydride monomer and fabricated by compression molding. Poly[trimellitylimidoglycine-co-1,6-bis(carboxyphenoxy)hexane] (TMA-gly:CPH) and poly[pyromellitylimidoalanine-co-1,6-bis(carboxyphenoxy)hexane] (PMA-ala:CPH) seem to be the most promising poly(anhydride-co-imides) for orthopaedic applications [Uhrich et al., 1995]. The compressive strength range of these copolymer is 30-60 MPa.
2.4.2 Degradation

Degradation of poly(anhydrides) is controlled through their backbone structure. Changes in the amount of aliphatic and aromatic monomers, sebacic acid (SA) and bis-(p-carboxyphenoxy)propane (CPP), respectively, can be used to control the rate of degradation [Attawia et al., 1995c]. However, unlike poly(α-hydroxy esters), these polymers degrade by surface erosion, not allowing anchorage dependent cells, such as osteoblasts, to attach, resulting in a lower number of attached cells as compared to PLGA disks after 24 hours [Attawia et al., 1995c]. The choice of anhydride greatly affects the
copolymer properties. More hydrophobic diacids, like CPP and CPH, have higher compressive strengths, while polymers of SA degrade have a much faster degradation rate [Uhrich et al., 1995]. For both (TMA-gly:CPH) and (PMA-ala:CPH) copolymers, the degradation rate was found to be constant over a period of two months and similar to the degradation rate of PLGA [Ibim et al., 1998]. However, degradation of the anhydride bond by hydrolysis occurred much faster than did the dissolution of the hydrolyzed products, leading to the formation of two distinct erosion zones, with erosion originating at the surface and moving to the center of the polymer samples [Seidel et al., 1996]. Degradation profiles for several poly(anhydride-co-imides) are shown in Figure 2-6 [Uhrich et al., 1997].

2.4.3 Biocompatibility

*In vitro*, rat calvaria osteoblasts have been used to assess the biocompatibility of these poly(anhydride-co-imides) through their ability to support cell adhesion and proliferation [Attawia et al., 1996]. This study has shown that there is no significant difference in initial adhesion of these cells between the polymer and TCPS. Phenotypic expression of osteoblasts has also been assessed by monoclonal antibodies against osteocalcin and osteopontin. *In vivo*, matrices of TMA-gly:CPH of 10:90 and 30:70 molar ratios exhibited responses similar to PLGA, while PMA-ala:CPH matrices had milder responses than those found in PLGA [Ibim et al., 1998]. Also, the toxicity of the monomers, TMA-gly, SA and CPH, has been studied. Addition of the monomer in the cell culture media did not result in morphological changes over a 21-day period [Attawia et al., 1995c].
Overall, both TMA-gly:CPH and PMA-ala:CPH copolymers have shown tissue compatibility similar to PLGA [Ibim et al., 1998].

**Figure 2-6.** Cumulative degradation, defined as percent aromatic monomers released, as a function of degradation time for varying molar ratios of (a) TMA-gly : CPH and (b) TMA-gly : SA in PBS at 37°C and pH = 7.4 [Uhrich et al., 1997]. Reproduced with permission from the publisher.
2.4.4 Functional Poly(anhydrides)

A photopolymerizable anhydride has been synthesized using methacrylate anhydride monomers to form highly crosslinked networks [Muggli et al., 1998]. Both methacrylated sebacic acid (SA-Me2) (Figure 2-7) and methacrylated 1,6-bis(carboxyphenoxy)hexane (CPH-Me2) have been synthesized for use in photopolymerization [Muggli et al., 1998]. Photoinitiators including Irgacure® 651 for UV wavelengths, and camphorquinone and ethyl-4-N,N-dimethylaminobenzoate for visible wavelengths have been investigated for crosslinking poly(anhydrides) [Anseth et al., 1997].

![Structure of methacrylated sebacic acid](image)

**Figure 2-7.** Structure of methacrylated sebacic acid

2.5 Poly(propylene fumarate)

In an attempt to improve solubility of highly crystalline poly(anhydrides), such as poly(fumaric acid), and facilitate polymer processing, anhydride monomers are copolymerized with aliphatic diols. The copolymerization of fumaric acid and propylene glycol leads to the formation of poly(propylene fumarate) (PPF) (Figure 2-8). The unsaturated fumarate C=C bond is utilized during the crosslinking of the polymer. One possible application is as a biodegradable scaffold for use in bone defects because this polymer is able to crosslink and it contains desirable ester groups. Crosslinked polymer
also has mechanical properties suitable for use as a temporary trabecular bone replacement [Gresser et al., 1996]. The antibiotics gentamicin and vancomycin have also been incorporated in a PPF-based composite without loss of mechanical properties [Gerhart et al., 1988b].

![Structure poly(propylene fumarate)](image)

**Figure 2-8.** Structure poly(propylene fumarate)

### 2.5.1 Synthesis

Several methods have been proposed for the synthesis of PPF which protect the fumarate double bond including: 1) transesterification of diethyl fumarate and propylene glycol [Peter et al., 1997a]; 2) condensation of propylene glycol and fumaric acid [Gerhart et al., 1988a]; 3) melt polymerization of bis(2-hydroxypropyl fumarate) trimer, synthesized from fumaric acid and propylene glycol in the presence of pyridine, and propylene bis(hydrogen maleate) trimer, synthesized from maleic anhydride and propylene glycol in toluene [Domb et al., 1996]; 4) direct esterification of fumaric acid and propylene glycol [Gresser et al., 1995]; and 5) transesterification of oligomer synthesized from fumaryl chloride and propylene glycol [Peter et al., 1999b; Peter et al., 1997c].

Using the above synthetic methods, a number average molecular weight range from 740 to 5000 Da was achieved, with the highest value attained by transesterification of the oligomer. In this scheme, potassium carbonate was used as a proton scavenger to
eliminate the addition of HCl and the acid catalyzed addition of alcohol groups across the fumaryl double bond during the synthesis of the oligomer [Peter et al., 1999b].

One possible method to initiate the *in situ* crosslinking reaction of PPF is similar to the clinically used free-radical polymerization of PMMA bone cement. Components of this system include N-vinyl pyrrolidinone (VP) as the crosslinking monomer, in conjunction with benzoyl peroxide, the initiator, and N,N-dimethyl-p-toluidine, the accelerator. This initiation system has been successfully used with PPF, minimizing the temperature rise and thus allowing crosslinking with minimal temperature rise [Peter et al., 1999a]. Incorporation of the monomer is an important factor to consider since VP is toxic to cells in high concentrations. Incorporation of the monomer depends on the polymer/monomer ratio, and is independent of the amount of accelerator used [Gresser et al., 1995]. For equal weights of PPF and VP, about 50% of the monomer is incorporated into the crosslinked network [Gresser et al., 1995].

### 2.5.2 Degradation

*In vitro* degradation studies of PPF/β-TCP have demonstrated an initial increase in strength and modulus over a twelve-week period [Peter et al., 1997b]. As shown in Figure 2-9, the compressive strength and compressive modulus range from 2-10 MPa and 60-250 MPa respectively for the PPF/β-TCP formulations. Without β-TCP, crosslinked PPF has maintained mechanical properties similar to trabecular bone through 7 weeks of degradation [Peter et al., 1997b]. When implanted subcutaneously in rats, the polymer formulations with β-TCP followed similar trends to the *in vitro* degradation rates with increasing strength and modulus as shown in Figure 2-10 [Peter et al., 1998]. However,
the polymers degrade more rapidly \textit{in vivo}. Other than an increase in mechanical strength, an additional advantage of β-TCP is its buffering capacity [Peter et al., 1998]. The formulations implanted \textit{in vivo} without β-TCP were mechanically unstable from the initial stages of implantation. \textit{In vitro} tests revealed that incorporation of β-TCP allowed no change in pH of PBS while omitting β-TCP resulted in a decrease in pH.

2.5.3 Biocompatibility

Biocompatibility of PPF/ β-TCP composite scaffolds was examined after subcutaneous implantation for a time period up to twelve weeks [Peter et al., 1998]. After an initial inflammatory response, a mature fibrous capsule encased the samples. Moreover, the scaffold osteoconductivity was assessed with a cavitational defect model in a rat [Yaszemski et al., 1995]. Significant bone ingrowth was evident for both prepolymerized and \textit{in situ} polymerized formulations 5 weeks post implantation with no evidence of inflammatory response.
Figure 2-9. Compressive strengths at yield and compressive moduli of PPF-based composites as a function of degradation time in PBS at 37°C and pH=7.4 [Peter et al., 1997b]. Error bars indicate ± SD for n = 5. Reproduced with permission from the publisher.
Figure 2-10. Compressive strengths at yield and compressive moduli of PPF-based composites implanted subcutaneously in male Lewis rats as a function of time [Peter et al., 1998]. Error bars indicate ± SD for n = 5. Reproduced with permission from the publisher.
2.5.4 Functional Poly(propylene fumarate)

A method of crosslinking low molecular weight PPF of about 750 Da number average molecular weight involved modification of the hydroxyl end groups [Domb et al., 1996]. PPF diol, when reacted with acryloyl chloride or epichlorhydrin, yields vinyl or epoxide functionalized end groups, eliminating the need for a crosslinking monomer. However, for this functionalized PPF, both VP and MMA were incorporated in the crosslinking formulation for greater mechanical strength [Peter et al., 1997a]. PPF has also been copolymerized with poly(ethylene glycol) through transesterification forming diblock and triblock copolymers [Suggs et al., 1998a; Suggs et al., 1997].

2.6 Summary

The ideal synthetic degradable polymer for orthopaedic application should satisfy the following design criteria: it should be biocompatible, have desired mechanical properties, degrade in a controlled fashion timed with tissue regeneration, have resorbable degradation products, be osteoconductive and osteoinductive, and allow for neovascularization. All the aforementioned polymers exhibit favorable osteoconductivity and biocompatibility and possess mechanical properties suitable for consideration for bone replacement. The advantage of poly(anhydrides) and poly(phosphazenes) lies in their versatility of functional groups. Polymer functionalization allows for crosslinking or covalent attachment of cell adhesion molecules that allow modulation of bone cell function and guided tissue regeneration. *In situ* crosslinking of unsaturated polymers
such as poly(propylene fumarate) can allow for a potential non-operative approach desirable for clinical problems. Design criteria for an ideal injectable material also includes the ability to crosslink *in situ* in a timely fashion without a large temperature rise and fill defects of various shapes and sizes.
CHAPTER 3

Objectives

The ultimate goal of this project was to develop a biodegradable material for bone tissue regeneration of a non-load bearing orthopaedic site. The intention was to design a biodegradable material that 1) can be formed in situ, 2) can reduce non-specific cell adhesion, 3) can be modified in the bulk with cell adhesive peptide ligands to promote specific receptor-ligand mediated osteoblast adhesion and migration, 4) can be formed into macroporous structures with an interconnected pore network for cell/tissue ingrowth and transport of nutrients and metabolic wastes, 5) can degrade in a controllable fashion, and 6) can allow for the differentiation of osteoprogenitor cells to the osteoblastic phenotype.

We hypothesized that in situ polymerizable hydrogels based on block copolymers of poly(propylene fumarate) (PPF) and poly(ethylene glycol) (PEG) would limit nonspecific cell adhesion dependent on the PEG block molecular weight. Incorporation of covalently linked RGDS peptide in the bulk of hydrogels would enhance the adhesion and migration of osteoblastic cells in a concentration-dependent manner. In addition, we hypothesized that hydrogels synthesized to three-dimensional macroporous structures would serve as a temporary biodegradable support for the culture of marrow stromal cells and allow for their differentiation to the osteoblastic phenotype under appropriate culture conditions.

The following 6 chapters test these hypotheses. In chapter 4, poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) copolymers were synthesized with terminal
poly(ethylene glycol) (PEG) blocks and their structure was verified with both chemical and physical techniques. Chapter 5 describes the synthesis of thin film hydrogels based on P(PF-co-EG) to examine the effect of PEG block length of the copolymer on non-specific cell adhesion. In addition, these thin film hydrogels were bulk-modified with RGDS peptide sequence utilizing a PEG spacer arm covalently linking the peptide to the backbone of the hydrogel. The effects of the ratio between the PEG block length of the copolymer and the PEG spacer arm length of the peptide and the concentration of the bulk-modified peptide on marrow stromal osteoblast adhesion, spreading, and migration were evaluated. The quantification of the surface ligand concentration of bulk-modified P(PF-co-EG) based hydrogels using biotin as a model ligand is described in chapter 6. Chapter 7 describes the synthesis of macroporous hydrogels using a factorial design to evaluate important factors for the control of the morphology of macroporous hydrogels. The degradation of these macroporous hydrogels was evaluated in chapter 8, where factors important for modulating the degradation profile of these materials were elucidated. Finally, in chapter 9, the differentiation of marrow stromal cells to the osteoblastic phenotype when cultured under conditions to promote that phenotype on RGDS bulk-modified macroporous hydrogels was evaluated.
CHAPTER 4

Synthesis of Triblock Copolymers of Methoxy Poly(Ethylene Glycol) and Poly(Propylene Fumarate)

Chapter Abstract

Amphiphilic block copolymers were synthesized by transesterification of hydrophilic methoxy poly(ethylene glycol) (mPEG) and hydrophobic poly(propylene fumarate) (PPF) and characterized. The copolymers synthesized with mPEG of molecular weights 570 and 800 had 1.9 and 1.8 mPEG blocks per copolymer, respectively, as measured by NMR, representing an ABA-type block copolymer. The number of mPEG blocks of the copolymer decreased with increasing mPEG block length to as low as 1.5 mPEG blocks for copolymer synthesized with mPEG of molecular weight 5190. At a concentration range of 5 to 25 wt% in phosphate buffered saline, copolymers synthesized with mPEG molecular weights of 570 and 800 possessed lower critical solution temperatures (LCST) between 40-45°C and 55-60°C, respectively. Aqueous solutions of copolymer synthesized with mPEG 570 and 800 also experienced thermoreversible gelation. The sol-gel transition temperature was dependent on the sodium chloride concentration as well as the mPEG block length. The copolymer synthesized from mPEG 570 had a transition temperature between 40°C and 20°C with salt concentrations between 1 and 10

wt%, while the sol-gel transition temperatures of the copolymer synthesized from mPEG molecular weight 800 were higher in the range of 75°C to 30°C with salt concentrations between 1 and 15 wt%. These novel thermoreversible copolymers are the first biodegradable copolymers with unsaturated double bonds along their macromolecular chain that can undergo both physical and chemical gelation and hold great promise for drug delivery and tissue engineering applications.

4.1 Introduction

Many developments have been made in creating improved biomaterials by careful modulation of the polymer or copolymer composition. One such development has been the copolymerization of poly(ethylene glycol) (PEG) with biodegradable polymers [Göpferich et al., 1999; Lucke et al., 2000; Song et al., 1999; Suggs et al., 1998a]. The incorporation of PEG, the most common moiety used to promote biocompatibility, has consistently improved the hydrophilic properties of the resulting copolymer [Suggs et al., 1998a; Suggs et al., 1999b; Tziampazis et al., 2000]. For example, diblock copolymers of methoxy poly(ethylene glycol) (mPEG) and poly(lactic acid) (PLA) exhibited significantly less protein adsorption and marrow stromal cell attachment compared to PLA homopolymers [Göpferich et al., 1999].

Block copolymers of poly(propylene fumarate) (PPF) and poly(ethylene glycol) (PEG) have been previously synthesized [Jo et al., 2000; Suggs et al., 1997]. Poly(propylene fumarate) is a biodegradable polyester which by itself has been
thoroughly characterized and investigated for orthopaedic applications [Peter et al., 1999b; Shung et al., 2002]. It consists of propylene fumarate units and can be crosslinked in situ via its fumarate double bonds. The copolymerization of PPF with PEG, resulted in a hydrophilic, biodegradable, biocompatible, and in situ crosslinkable copolymer [Jo et al., 2000; Suggs et al., 1998a; Suggs et al., 1998b; Suggs et al., 1999a; Suggs et al., 1997; Suggs et al., 1999b]. The poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) copolymer has been examined for use in cardiovascular applications. Suggs et al. reported a significant decrease in platelet adhesion measured under flow conditions on the copolymer when compared to PPF [Suggs et al., 1999c]. Using an in vivo rat cage implant system, P(PF-co-EG) exhibited an exudate leukocyte concentration comparable to that of the negative control [Suggs et al., 1999b].

Biodegradable copolymers incorporating PEG can possess unique thermoreversible properties, depending on the hydrophobic block structure and PEG block length. Copolymers consisting of PEG and poly(α-hydroxy esters) possess lower critical solution temperatures (LCST) and sol-gel transition temperatures dependent on the PEG block length [Jeong et al., 2000; Jeong et al., 1999b]. These copolymers undergo gelation in water upon reaching their LCST and have thermoreversible properties suitable for drug delivery systems. Another class of biodegradable copolymers, poly(organophosphazene) incorporating mPEG, also possess a LCST [Lee et al., 1999; Song et al., 1999]. Lee et al. showed decreased LCST of the poly(organophosphazene) based copolymers with increasing sodium chloride concentration. However, unlike copolymers synthesized with a PPF block, these thermoreversible copolymers do not offer further chemical crosslinking.
In this study, we investigated the synthesis of ABA-type block copolymers of PPF and mPEG of variable mPEG block molecular weights. In addition, we examined the thermoreversible properties of the block copolymers as a function of the molecular weight of the PEG block.

4.2 Materials and Methods

4.2.1 Materials

Diethyl fumarate (Acros, Pittsburgh, PA), propylene glycol (Fisher, Pittsburgh, PA), and methoxy poly(ethylene glycol) (mPEG) of four nominal molecular weights of 550, 750, 2000, and 5000 (Aldrich, Milwaukee, WI) were used as received. One molar anhydrous zinc chloride in diethyl ether and hydroquinone were purchased from Sigma and used as received. All solvents used for purification were acquired from Fisher as reagent grade. Sodium chloride (Fisher) and phosphate buffered saline (PBS) (GibcoBRL, Grand Island, NY) were purchased and used as received. Trifluoroacetic anhydride was purchased from Sigma.

4.2.2 Polymer Synthesis

Poly(propylene fumarate) (PPF), the hydrophobic block, was synthesized as previously described [Shung et al., 2002]. Briefly, diethyl fumarate, propylene glycol, zinc chloride, and hydroquinone were combined in a three-neck round bottom flask in a 1:3:0.01:0.003 molar ratio. Zinc chloride was added as a catalyst while the hydroquinone
was added to prevent spontaneous crosslinking during the transesterification reaction. The contents of the reactor were then mixed using an overhead mechanical stirrer. This mixture was heated to a temperature of 150\(^\circ\)C under a nitrogen purge to form a diester intermediate, bis(2-hydroxypropyl) fumarate, and the byproduct, ethanol, which was removed by distillation. The diester intermediate was then transesterified under vacuum (< 1 mmHg) at a maximum temperature of 150\(^\circ\)C for 3 hours to form the linear unsaturated PPF product and propylene glycol. The number average molecular weight of the PPF was determined by gel permeation chromatography (GPC) and nuclear magnetic resonance (NMR) spectroscopy.

The reaction scheme of the ABA-type PPF-PEG block copolymer synthesis is illustrated in Figure 4-1. PPF prepolymer of the same molecular weight was used for each copolymerization reaction. Copolymers were synthesized with methoxy poly(ethylene glycol) (mPEG) nominal molecular weights of 550, 750, 2000, and 5000. Number average molecular weight of the mPEGs was measured by NMR. The mPEG was added to the flask in a 1:2 PPF to mPEG molar ratio. The transesterification reaction was carried out under vacuum (< 1 mmHg) at a reaction temperature of 160\(^\circ\)C. The reaction was continued until the depletion of the starting prepolymer and the formation of ABA-type block copolymers as evidenced by GPC.

To purify the copolymer, the resulting product was dissolved in dichloromethane, filtered, and precipitated into ethyl ether under vigorous stirring. Zinc chloride and hydroquinone, which are soluble in ethyl ether, were removed in the precipitation process. Copolymers synthesized were either waxy or solid in ethyl ether. For waxy copolymers, the ethyl ether was decanted and any remaining solvent was removed by
rotoevaporation. Powdery copolymers were isolated by filtration and subsequently dried under vacuum at room temperature to remove residual organic solvent.

4.2.3 Gel Permeation Chromatography

To determine the molecular weight distribution of the resulting polymers and copolymers, GPC was performed with a differential refractometer (Waters, Milford, MA). Chloroform was used as an eluent at a flow rate of 1 ml/min through a Phenogel guard column (50 x 7.8 mm, 5 μm, Phenomenex, Torrance, CA) and

![Poly(Propylene Fumarate) and Methoxy Poly(Ethylene Glycol)](image)

**Figure 4-1.** Reaction scheme for the copolymerization of poly(propylene fumarate) and methoxy poly(ethylene glycol).
a Styrage HR4E column (300 x 7.8 mm, 5 μm, Waters, Milford, MA). Polystyrene standards were used to construct a calibration curve. Molecular weights reported are relative to the polystyrene calibration.

The average number of PEG blocks within the copolymer was calculated from the following equation [Yan et al., 1987]:

\[
b = \frac{M_{n,CP} - M_{n,PPF}}{M_{n,mPEG}} \quad (1)
\]

where \( b \) is the number of PEG blocks with \( b=2 \) suggesting an ABA block copolymer structure and \( b=1 \) suggesting an AB block copolymer structure; and, \( M_{n,CP} \), \( M_{n,PPF} \), and \( M_{n,mPEG} \) are the number average molecular weights of the copolymer, PPF, and mPEG respectively.

### 4.2.4 Nuclear Magnetic Resonance Spectroscopy

NMR measurements were performed with a 400 MHz spectrometer (Bruker, Zurich, Switzerland) using 1 wt % solutions in CDCl₃. Number average molecular weight of PPF was determined by end group analysis [Jo et al., 2000]. Molecular weight of PPF was determined by reacting the hydroxyl end groups with trifluoroacetic anhydride (TFA) causing a downfield shift in the terminal methyl protons.

Integration of the proton peaks from the copolymers was used to determine the number average molecular weights of the block copolymers. The number average molecular weights of the mPEG were calculated by taking the ratio of the integrated peak of the end methoxy group protons to the PEG methylene protons from the mPEG NMR
spectrum. The number of mPEG blocks was determined from end-group analysis after derivatizing end hydroxyl groups with TFA. The number average molecular weight of the PPF block was determined from the number of mPEG blocks and the integrated proton peak ratio of the end methoxy group of mPEG to the PPF methyl protons.

4.2.5 Fourier Transform Infrared Spectroscopy

Infrared (IR) spectra were obtained using a Nicolet 550 spectrometer (Madison, WI). The samples were heated until melted and cast onto a zinc selenide attenuated total reflection crystal forming a thin film on the crystal at room temperature. Thirty-two scans were taken at a resolution of 4 cm\(^{-1}\) under a dry nitrogen purge.

4.2.6 Lower Critical Solution Temperature Determination

To determine the lower critical solution temperature (LCST), aqueous solutions of 25, 15, and 5 wt % copolymer were prepared in phosphate buffered saline (PBS), pH = 7.4 by dissolving the copolymer at 4°C overnight. One ml of the solution was placed into 1.5 ml cuvettes and immersed in a water bath. The temperature was raised 5°C every 20 min between the range of 5°C to 75°C. At each temperature, the absorbance at 500 nm was measured using a Hewlett Packard 8452A Diode Array UV-Vis Spectrophotometer (Waldbonn, Germany). The LCST was defined as the temperature where at least 50% of the absorbance change occurred [Song et al., 1999].
4.2.7 Sol-Gel Transition Temperature Determination

The thermally induced sol-gel transition temperature was determined by the test-tube inversion method [Jeong et al., 1999a]. Aqueous solutions (1 ml) of 25 wt% copolymer and 1.0, 2.5, 5.0, 10, or 15 wt% sodium chloride were prepared in a 9 mm diameter test tube and equilibrated at 4°C overnight. Test tubes were then placed in a temperature controlled water bath. The temperature was increased in 2°C increments and held constant for 15 min. The gelation temperature was characterized by the formation of a firm gel that remained intact when the tube was inverted 180° [US Pharmacopoeia, 2000].

4.3 Results and Discussion

4.3.1 Gel Permeation Chromatography

The block copolymers containing hydrophilic PEG blocks were obtained by the transesterification reaction of PPF with mPEG (Figure 4-1). Prior to the transesterification reaction synthesis, the number average molecular weight ($M_n$) of the PPF was determined by GPC to be 1620 with a polydispersity index (PI) of 1.5. Copolymers CP550 and CP750, synthesized with mPEG of nominal molecular weight 550 or 750 respectively, were waxy while copolymers CP2000 and CP5000, synthesized from mPEG of nominal molecular weight 2000 or 5000, were powdery at room temperature.

GPC was utilized to measure the $M_n$ and PI of the starting mPEG and the resulting copolymers relative to polystyrene standards (Table 4-1). In all cases, the
chromatographs of the copolymers indicated the absence of the initial prepolymer, PPF and mPEG, which suggested a successful copolymerization. There was a decrease in the retention time, corresponding to an increase in the molecular weight, of the copolymer when compared to the mPEG and PPF. The average numbers of mPEG blocks were calculated, and CP550 and CP750 were determined to be ABA-type block copolymers by GPC. Both CP550 and CP750 were copolymerized for 4 hours; CP2000 was copolymerized for 12 hours; CP5000 was copolymerized for 16 hours. The GPC chromatographs of CP2000 as well as the starting polymers are shown in Figure 4-2. With increasing reaction time, there was an increase in the yield of ABA-type block copolymer formed. CP5000 revealed a well resolved chromatograph with two peaks corresponding to diblock (AB) and triblock (ABA) copolymer, respectively.

Table 4-1. Number average molecular weight ($M_n$) and polydispersity index (PI) of the mPEG prepolymer and respective copolymers as determined by GPC relative to polystyrene standards. *GPC chromatographs were bimodal and the number of mPEG blocks could not be determined.

<table>
<thead>
<tr>
<th>Copolymer Formulation</th>
<th>$M_n$, mPEG</th>
<th>PI$_{mPEG}$</th>
<th>$M_n$, CP</th>
<th>PI$_{CP}$</th>
<th>Number of mPEG blocks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP550</td>
<td>1090</td>
<td>1.10</td>
<td>4000</td>
<td>1.39</td>
<td>2.1</td>
</tr>
<tr>
<td>CP750</td>
<td>1440</td>
<td>1.09</td>
<td>4450</td>
<td>1.24</td>
<td>2.0</td>
</tr>
<tr>
<td>CP2000</td>
<td>3820</td>
<td>1.12</td>
<td>9430</td>
<td>1.39</td>
<td>*</td>
</tr>
<tr>
<td>CP5000</td>
<td>11010</td>
<td>1.10</td>
<td>18500</td>
<td>1.53</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 4-2. GPC chromatographs of (a) PPF prepolymer, (b) mPEG prepolymer, and (c-e) CP2000 block copolymers after (c) 4 hours, (d) 8 hours, and (e) at the completion of the reaction. *ABA block copolymer. **AB block copolymer.
A previous method for the transesterification of PPF and PEG produced a mixture of copolymers, mostly diblock with unreacted prepolymer [Suggs et al., 1997]. The use of PEG with two hydroxyl end groups instead of mPEG renders the formation of BAB block as well as multi-block copolymers, with PEG as a middle block [Gallardo et al., 1998]. An ABA-type PPF-PEG copolymer has been previously synthesized in our laboratory by organic reaction between PPF and bis-carboxymethyl PEG. However, the method was limited by the use of a multi-step reaction and availability of end functionalized PEG [Jo et al., 2000]. In this study, copolymers were synthesized from PPF and monofunctional mPEG. Only one reactive end of the PEG was available for the one-step transesterification reaction allowing for an ABA-type block copolymer, with a maximum number of three blocks. GPC data showed that with increased mPEG molecular weight there was a decrease in the number of mPEG blocks in the copolymer. CP550 and CP750 were found to be ABA-type block copolymers, whereas CP2000 and CP5000 contained decreasing amounts of ABA-type block copolymers.

For CP2000 and CP5000, diffusional limitations may have been a factor in the copolymer synthesis as reported in the PPF synthesis [Shung et al., 2002]. The copolymerization reaction for the four formulations was carried out at the same temperature of 160°C. However, increasing the molecular weight of the mPEG increased the viscosity of the reaction mixture. The increased viscosity may have prevented the diffusion of propylene glycol out of the reaction solution, which is necessary to drive the copolymerization forward. A further explanation of the decrease in the number of mPEG blocks for higher molecular weight mPEGs could be due to steric hindrances caused by the longer mPEG chains.
4.3.2 Nuclear Magnetic Resonance Spectroscopy

The $M_n$ of the PPF prepolymer was measured using end-group analysis by derivatizing the hydroxyl end groups with trifluoroacetic anhydride (TFA) and determined to be 1570. The mPEG molecular weights determined by NMR were comparable to their respective nominal molecular weights (Table 4-2).

The chemical structure of the copolymer was correlated to the $^1$H NMR spectrum (Figure 4-3). The ratio between the integrated peaks of the fumarate and methyl protons of PPF in the copolymer ranged from 2-2.96 to 2-3.10 fumarate protons to methyl protons which is comparable to the calculated ratio of 2-3 based on the polymer structure. The similarity of the measured fumarate proton to methyl proton ratio for the PPF block with the calculated value suggested minimal additional reaction to the double bond, a limiting factor for the synthesis of unsaturated polymers.

The number of mPEG blocks in the copolymer was calculated by end-group analysis by comparing the protons of the end hydroxyl groups present in uncopolymerized PPF with the protons of the end methyl groups of mPEG (Table 4-2). An increase in mPEG molecular weight resulted in a decrease in incorporated mPEG blocks. The number average molecular weight of the PPF block for CP550 and CP750 was 1660 and comparable to that of the PPF prepolymer, 1570, indicating that the PPF block molecular weight remained unchanged at the end of the transesterification reaction. Utilizing NMR, the ABA block structure for CP550 and CP750 was confirmed, with 1.9 and 1.8 mPEG blocks per polymer chain respectively. A decrease in the PPF block length for CP2000 and CP5000 compared to the PPF prepolymer was observed suggesting a mixture of AB
and ABA-type block copolymer. This decrease in PPF block length could be due to an increased number of PPF chains compared to mPEG chains, representing a mPEG:PPF block molar ratio greater than 2:1.

Table 4-2. Molecular weight of the mPEG prepolymer and mPEG and PPF blocks as determined by $^1$H NMR.

<table>
<thead>
<tr>
<th>Copolymer Formulation</th>
<th>mPEG nominal molecular weight</th>
<th>$M_n$ mPEG Block</th>
<th>$M_n$ PPF Block</th>
<th>$M_n$ CP</th>
<th>Number of mPEG blocks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP550</td>
<td>550</td>
<td>570</td>
<td>1660</td>
<td>2730</td>
<td>1.9</td>
</tr>
<tr>
<td>CP750</td>
<td>750</td>
<td>800</td>
<td>1660</td>
<td>3120</td>
<td>1.8</td>
</tr>
<tr>
<td>CP2000</td>
<td>2000</td>
<td>1960</td>
<td>1430</td>
<td>4760</td>
<td>1.7</td>
</tr>
<tr>
<td>CP5000</td>
<td>5000</td>
<td>5190</td>
<td>1340</td>
<td>9170</td>
<td>1.5</td>
</tr>
</tbody>
</table>
Figure 4-3. Representative $^1$H NMR spectrum of the block copolymer CP750 in CDCl$_3$. 
4.3.3 Fourier Transform Infrared Spectroscopy

The IR spectra of the copolymers showed characteristic PPF bands [Peter et al., 1999b]: carbonyl stretching at 1720 cm\(^{-1}\) and C=C stretching at 1640 cm\(^{-1}\) (Figure 4-4). Additionally, for all copolymers, IR spectra provided supporting evidence of near complete copolymerization. Qualitatively, the substantial reduction of the strong band above 3150 cm\(^{-1}\) was attributed to the near complete reaction of the mPEG hydroxyl end groups with PPF suggesting an ABA-type block copolymer structure with mPEG end blocks.

![Representative FTIR spectra](image)

**Figure 4-4.** Representative FTIR spectra of the (a) CP2000 block copolymer, (b) mPEG prepolymer, and (c) PPF prepolymer.
Table 4-3. LCST determined by UV/VIS for P(PF-co-EG) with varying mPEG block lengths and solution concentrations. Thermal properties shown for 5, 15, and 25 wt % copolymer solutions in PBS at pH 7.4. *No LCST was observed below 75°C.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>wt % copolymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation</td>
<td>5%</td>
</tr>
<tr>
<td>CP550</td>
<td>40</td>
</tr>
<tr>
<td>CP750</td>
<td>55</td>
</tr>
<tr>
<td>CP2000</td>
<td>*</td>
</tr>
<tr>
<td>CP5000</td>
<td>*</td>
</tr>
</tbody>
</table>

4.3.4 Lower Critical Solution Temperature

The thermoreversible properties of copolymer solutions were evaluated by measuring the LCST as a function of the copolymer concentration in PBS. These amphiphilic copolymers, comprised of maximum two hydrophilic blocks, mPEG, and a hydrophobic block, PPF, exhibited thermoreversible properties.

The mPEG block length was a critical parameter in determining the LCST. Two of the copolymers tested, CP550 and CP750, possessed a LCST between 40°C and 60°C in PBS, with CP550 exhibiting the lower LCST (Table 4-3). An increase of approximately 200 Da in the mPEG molecular weight increased the LCST by 20°C. Similar trends were observed with other thermoreversible copolymers, where the LCST was dependent on the PEG molecular weight [Jeong et al., 1999a; Song et al., 1999]. In PBS, CP2000 and CP5000, which contain larger molecular weight mPEG, showed no measurable difference in UV-Vis absorbance below 75°C. These copolymers may be too hydrophilic to possess a LCST in the tested temperature range. Additionally, CP550 and CP750 had LCSTs that
were not highly dependent on the concentration in the range of 5 to 25 wt% copolymer and varied only by 5°C as observed for other thermoreversible copolymers in the concentration range tested [Jeong et al., 1999a; Song et al., 1999].

4.3.5 Sol-Gel Transition Temperature

The effect of salt concentration on the thermoreversibility of the P(PF-co-EG) copolymers CP550 and CP750, which exhibited LCST, was examined by measuring the sol-gel transition temperature in aqueous solutions of sodium chloride (Figure 4-5). An inverse relationship between sodium chloride concentration and gelation temperature was observed for both copolymers. For CP550, the sol-gel transition temperature ranged from 45°C to 20°C for salt concentrations of 1 to 10 wt%. For CP750, the transition temperature ranged from 75°C to 30°C for salt concentrations between 1 and 15 wt%.

Poly(ethylene glycol) (PEG) copolymers are typically thermoreversible. This thermoreversibility is derived from hydrogen bonding interactions between PEG and water molecules [Kjellander et al., 1981]. Aqueous solutions of various salts have been used previously to modulate the LCST [Jorgensen et al., 1997; Lee et al., 1999; Von Hippel et al., 1969]. Addition of sodium chloride into the solution alters hydrogen bonding, resulting in the observed decrease in both LCST and gelation temperature.

Compared to other biodegradable thermoreversible copolymers such as those synthesized from PLA and poly(organophosphazene), block copolymers with PPF and mPEG may have the advantage in that they can also be chemically crosslinked. The fumarate double bond of the PPF can be covalently crosslinked using free-radical
initiation [Jo et al., 2001; Peter et al., 1999a]. Thus, the mechanical properties of the gel can be conceivably controlled by both physical and chemical gelation.

Figure 4-5. The effect of salt concentration on the sol-gel transition temperature of CP550 (■) and CP750 (square) block copolymers. Error bars represent means ± standard deviation for n=3.
4.4 Conclusion

Block copolymers with ABA-type block structure were synthesized from PPF and mPEG utilizing a simple transesterification procedure. Four block copolymers were synthesized with a 2:1 mPEG:PPF molar ratio and mPEGs of molecular weights 570, 800, 1960 and 5190 and PPF of molecular weight 1570 as determined by NMR. The use of mPEG ensured that only one PEG block attached to each end of the PPF chain. The conversion of the mPEG and PPF to the ABA copolymer was dependent on the mPEG molecular weight, with decreased conversion for increasing mPEG molecular weight in the range of 1.9 to 1.5 mPEG blocks. ABA copolymers synthesized with mPEG of 570 and 800 number average molecular weight showed thermoreversible properties that were dependent on the mPEG molecular weight and salt concentration. Furthermore, the hydrophobic PPF block offers the added advantage of being highly unsaturated and chemically crosslinkable.
CHAPTER 5

Adhesion and Migration of Marrow-Derived Osteoblasts on Injectable in Situ Crosslinkable Poly(propylene fumarate-co-ethylene glycol)-Based Hydrogels with a Covalently Linked RGDS Peptide

Chapter Abstract

Marrow-derived osteoblasts were cultured on poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) based hydrogels modified in bulk with a covalently linked RGDS model peptide. A poly(ethylene glycol) spacer arm was utilized to covalently link the peptide to the hydrogel. Three P(PF-co-EG) block copolymers were synthesized with varying poly(ethylene glycol) block lengths relative to poly(ethylene glycol) spacer arm. A poly(ethylene glycol) block length of nominal molecular weight 2000 and spacer arm of nominal molecular weight 3400 were found to reduce non-specific cell adhesion and show RGDS concentration dependent marrow-derived osteoblast adhesion. A concentration of 100 nmol/ml RGDS was sufficient to promote adhesion of 84 ± 17% of the initial seeded marrow-derived osteoblasts compared to 9 ± 1% for the unmodified hydrogel after 12 hrs. Cell spreading was quantified as a method for evaluating adhesivity of cells to the hydrogel. A megacolony migration assay was utilized to assess the migration characteristics of the marrow-derived osteoblasts on RGDS modified

hydrogels. Marrow–stromal osteoblasts migration was greater on hydrogels modified with 100 nmol/ml linked RGDS when compared to hydrogels modified with 1000 nmol/ml linked RGDS, while proliferation was not affected. These P(PF-co-EG) hydrogels modified in the bulk with RGDS peptide are potential candidates as in situ forming scaffolds for bone tissue engineering applications.

5.1 Introduction

The choice of biomaterials for a specific application has been dependent on available materials used from other fields or designed to be inert with little interaction with the host tissue. Recently, researchers have concentrated their efforts towards the synthesis of biologically inspired materials (BIMs) that interact in a specific manner with host tissue.[Dee et al., 1998; Hern et al., 1998; Jo et al., 2000; Kantlehner et al., 1999; McClary et al., 2000; Nuttelman et al., 2001; Rezania et al., 1999a; Tziampazis et al., 2000] One approach to BIMs has been to covalently link peptide sequences derived from extracellular matrix (ECM) proteins to synthetic inert biomaterials. On a cellular level, BIMs facilitate a specific interaction between cell and biomaterial via cell receptors and ligands linked to the biomaterial substrate.

There have been a variety of polymers utilized as biomaterials including poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), a versatile water-soluble block-copolymer which has the potential to be crosslinked in situ into thin films [Suggs et al., 1999a] and macroporous tissue engineering scaffolds [Behravesh et al., 2002a]. This
block copolymer is composed of poly(propylene fumarate), a biodegradable highly unsaturated hydrophobic polymer capable of being crosslinked in situ, and poly(ethylene glycol) (PEG), a hydrophilic polymer imparting water solubility to the resulting block copolymer. In vivo, P(PF-co-EG) hydrogel films have been shown to be biocompatible, allowing for intimate contact with surrounding tissue with a minimal fibrous encapsulation [Suggs et al., 1998b; Suggs et al., 1999b].

Cell adhesion and spreading is necessary for viability of anchorage-dependent cells and required for subsequent cell functions including proliferation and differentiation [Hynes, 1992; Ranucci et al., 2001; Rezania et al., 1999a]. For BIMs, a reduction in non-specific cell adhesion combined with covalent attachment of ECM segments allows for the specific desired adhesion of a cell via receptor-ligand interactions. Research has shown that many cells can adhere to surfaces modified with cell binding domains of ECM proteins. Of the ECM protein segments, the most widely studied has been the arginine-glycine-aspartic acid (RGD) sequence [Dee et al., 1996; Hern et al., 1998; Kantlehner et al., 1999; Massia et al., 1991; Pierschbacher et al., 1984; Puleo D.A., 1991]. ECM cell binding domains can interact with integrins, a family of trans-membrane proteins linked to the cell’s internal architecture [Anselme, 2000; Hynes, 1992]. This integrin-peptide, or receptor-ligand, bond allows the cell to adhere to the substrate with the strength of adhesion dependent on the strength of each bond and the total number of bonds.

In addition to cell adhesion, researchers have postulated a relationship between cell-substrate adhesivity and speed of cell movement [Lauffenburger et al., 1993; Webb et al., 2000]. A bimodal relationship has been found for many cell types, where cell speed is
high at intermediate cell-substrate adhesivities, and negligible at both high and low cell-substrate adhesivities [Lauffenburger et al., 1993]. Consideration of cell migration from surrounding tissue into the site of a defect can be an important factor for many applications [Malmquist, 1999; Zygourakis, 1996]. For example, osteoblast migration to a bone defect may ultimately lead to bone formation and repair [Anselme, 2000; Attawia et al., 1995b; Jones et al., 1977].

*In situ* formed BIMs for bone tissue engineering must be modified in the bulk. For PEG based hydrogels, availability of the peptides to the cells has been shown to be dependent on the relationship of the molecular weight of the PEG spacer arm of the peptide sequence and the molecular weight of the surrounding PEG chains. In this study, the peptide sequence RGDS was selected as a model peptide in an effort to investigate the effect of (1) the PEG block length of P(PF-co-EG) block copolymer without peptide modification on adhesion, (2) the PEG block length of P(PF-co-EG) block copolymer compared to the length of the spacer arm of the covalently linked RGDS peptide on adhesion, and (3) the concentration of the covalently linked RGDS peptide on the adhesion and migration of marrow-derived osteoblasts.
5.2 Material and Methods

5.2.1 Materials

Poly(ethylene glycol) diacrylate (PEG-DA) was purchased from Aldrich Chemical (Milwaukee, WI) with a nominal molecular weight of 700. Ammonium persulfate (APS) (Acros, Pittsburgh, PA) and L-ascorbic acid (AH) (Aldrich Chemical) were purchased and used as received.

Poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) block copolymers were synthesized as previously described [Behravesh et al., 2002b]. Briefly, using a three neck round bottom flask with overhead mechanical stirring, bis-(2-hydroxy propyl) fumarate was synthesized under a nitrogen atmosphere by a reaction of diethyl fumarate (Acros) and propylene glycol (Fisher, Pittsburgh, PA) at 160°C with zinc chloride (Sigma, St. Louis, MO) and hydroquinone (Sigma). The product was then transesterified under vacuum (<1 mmHg) at 150°C to form poly(propylene fumarate) (PPF). Progression of the molecular weight of the PPF was monitored every hour by gel permeation chromatography (GPC) until a number average molecular weight of 1700 (PI=1.8) was achieved (based on a calibration curve with polystyrene standards).

Three P(PF-co-EG) block copolymers were then synthesized by the transesterification reaction of PPF and methoxy poly(ethylene glycol) (mPEG) (Aldrich Chemical) of nominal molecular weights of 750, 2000, and 5000 corresponding to number average molecular weights of 800, 1960 and 5190 (measured by nuclear magnetic resonance spectroscopy). The extent of the transesterification was monitored by GPC and continued until the depletion of the starting materials. The copolymer was then dissolved
in methylene chloride (Fisher) and purified by precipitation in ethyl ether (Fisher). Copolymers were either waxy or powdery at room temperature. Ethyl ether was decanted for a waxy copolymer and any remaining solvent was removed by rotoevaporation. A powdery copolymer was isolated by filtration. Copolymers were subsequently dried under vacuum at room temperature to remove any residual organic solvent.

Acryloyl-poly(ethylene glycol)-GRGDS and acryloyl-poly(ethylene glycol)-GRDGS were synthesized from acryloyl-poly(ethylene glycol) N-hydroxysuccinimide (Ac-PEG-NHS) (Shearwater, Huntsville, AL) of PEG nominal molecular weight of 3400 and the oligopeptide sequences GRGDS and GRDGS (Bachem, Torrance CA), respectively [Hern et al., 1998]. Ac-PEG-NHS and the oligopeptide were dissolved separately in 5 mM sodium bicarbonate buffer, pH 8.2, at a final concentration of 75 mg/ml and 1 mg/ml, respectively. A molar ratio of 2 Ac-PEG-NHS to peptide was used to minimize loss of peptide by ensuring a complete reaction of the GRGDS peptide. Ac-PEG-NHS was added drop-wise to the peptide solution with vigorous stirring and allowed to react for 2 hrs. Low molecular weight by-products and sodium bicarbonate were removed via dialysis using a molecular weight cut-off dialysis membrane of 2000 (Spectrum Laboratories, Rancho Dominguez, CA) with 5 double distilled deionized water (DDW) changes over the course of 2 days. The Ac-PEG-peptides were lyophilized to remove water for 48 hours and stored below -4°C until use.

Cell culture grade reagents included phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA), Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen), fetal bovine serum (FBS) (Gemini Bio-product, Calabasas, CA), antibiotic/antimycotic (Invitrogen),
and trypsin/EDTA (Sigma). PicoGreen DNA quantification kit was purchased from Molecular Probes (Eugene, OR).

5.2.2 Hydrogel Fabrication and Characterization

P(PF-co-EG) was mixed with PEG-DA on a 1:1 wt% basis and dissolved with vigorous mixing in DDW in a final polymer concentration of 66 wt%. Subsequently, Ac-PEG-peptide was mixed and dissolved in the polymer solution, the concentration of which was determined to result in a selected peptide concentration after equilibrium swelling. The polymer solution was centrifuged to remove small bubbles trapped within the aqueous solution. APS and AH were added consecutively at final concentrations of 0.01 M and gently mixed after each addition. A typical reaction consisted of 2 g P(PF-co-EG), 2 g PEG-DA, 0-60 mg Ac-PEG-peptide, 1.8-1.9 ml DDW, 57 µl APS (1 M), and 57 µl AH (1 M). The solution was cast between two glass plates with a 0.5 mm spacer and allowed to crosslink overnight at 37°C. Hydrogels were submerged and swollen in PBS for 6-8 hours and cut with a #15 corks borer (21 mm diameter) to fit in 12 well tissue culture plates in their swollen state.

To measure the equilibrium water content (EWC), disk shaped hydrogels were swollen in PBS for at least 12 hours, weighed (Wₜ), and dried under vacuum overnight. Subsequently, the weight of the dried hydrogels was recorded (Wₜ). EWC was calculated according to the following equation:

\[
EWC = \frac{Wₜ - Wₜ}{Wₜ} \cdot 100
\]
A captive air bubble technique was used to measure the air-in-water static contact angle. Swollen hydrogels were attached by 1 drop of cyanoacrylate to a glass plate connected to a holding rod and inverted into PBS. A small bubble (approximately 5 μl in volume) was formed underneath the hydrogel via a curved needle and the static contact angle of each formulation was measured at least 5 times using a NRL contact angle goniometer (Rame-Hart, Mountain Lakes, NJ).

5.2.3 Marrow-Derived Osteoblast Isolation and Culture

Marrow-derived osteoblasts were isolated as previously described [Maniatopoulos et al., 1988]. Briefly, femurs and tibias of 125-149 g male Wistar rats (Harlan, Indianapolis, IN) were excised aseptically. Bone marrow was flushed out using cell culture media expelled from an 18 1/2 gauge needle. Cell culture suspensions were prepared by repeated aspiration of the cells through an 18 1/2 gauge followed by a 22 1/2 gauge needle. The cell suspension from two animals was seeded into one triple flask having a surface of 500 cm² and cultured in DMEM with 10% fetal bovine serum and 1% antibiotics. After 2 days in culture, non-adherent cells were removed along with the media. Subsequently, all cell culture was conducted with the above media with included osteogenic supplements: 10⁻⁸ M dexamethasone, 10⁻³ M β-glycerol phosphate, and 50 mg/l L-ascorbic acid to induce the osteoblastic phenotype of the marrow stromal cells [Göpferich et al., 1999; Ishaug et al., 1997].

After one week of cell culture, the cells were enzymatically lifted with 0.05% Trypsin/0.53mM EDTA exposure. The cells were then cryopreserved in FBS with 10% DMSO in liquid nitrogen until use at a concentration of 3x10⁶ cells per ml. When called
for, cells were thawed (viability was assessed by a Trypan Blue exclusion method [Freshney, 1987]), and cultured using media with osteogenic supplements at a concentration of $10^6$ cells per T-75 culture flasks (passage 1). Media was changed every 3 days. Prior to reaching confluency, cells were washed two times with PBS, lifted by exposure to 2 ml of Trypsin/EDTA solution for 5 min, neutralized with 2 ml of FBS, diluted with DMEM, centrifuged, and resuspended for use in the study. All experiments conducted utilized cells at this 2nd passage.

5.2.4 Marrow-Derived Osteoblast Adhesion on P(PF-co-EG) Hydrogels

Cell adhesion was quantified utilizing a sedimentation assay. Swollen hydrogels, which were cut with a #15 cork borer (21 mm in diameter) to fit 12 well tissue culture plates, were transferred to 70% ethanol overnight and subsequently placed in 50 ml PBS and washed 5 times during a 48 hr period to remove ethanol prior to cell culture. Each film was submerged in PBS in a 12-well tissue culture plate. A steel annulus (inner diameter of 15 mm) was placed on top to stabilize the hydrogel and allow for a uniform hydrogel area of approximately 1.8 cm$^2$ for cell seeding. Suspended marrow-derived osteoblasts (250 μl) were seeded onto the hydrogels at a concentration of 40,000 cells per well, approximately 40% of the number of cells necessary to reach confluency (53,000 cells per cm$^2$) [Ishaug et al., 1994].

Two types of controls were utilized to assess the specificity of cell adhesion to the RGDS peptide sequence: 1) use of hydrogels with a covalently-linked scrambled RDGS peptide and 2) use of marrow-derived osteoblasts incubated with soluble GRGD in
DMEM. The soluble GRGD was mixed in the cell suspension media at a concentration of 5 mM. Cells were incubated for 30 min before seeding onto hydrogel films.

Cells were placed in an incubator for 12 hrs and then washed 2 times with 1 ml of PBS applied to the side of the well using a micro-pipetter. Two ml of sterile DDW were added to each well. A freeze thaw cycle followed by sonication was utilized to suspend DNA into solution. PicoGreen analysis of double stranded DNA was performed in 96-well plates using an automated plate reader (FLx 800, Bio-Tek Instruments, Winooski, VT) according to the manufacturer’s instructions. Measured fluorescence intensities were correlated to the number of cells using a calibration curve made with marrow-derived osteoblasts of known concentration in the range of 0 to 55,000 cells per ml.

5.2.5 Marrow-Derived Osteoblast Migration and Proliferation on P(PF-co-EG) Hydrogels

A megacolony migration assay was utilized to measure the change in surface area of the hydrogel covered by the marrow-derived osteoblasts as a function of covalently linked RGDS concentration. Hydrogels were prepared as before for the adhesion experiments in the 12 well tissue culture plates. A second annulus with an inner diameter of 7 mm was placed within the first annulus. The purpose of this second annulus was to isolate the cells in the center of the hydrogel film.

Marrow-derived osteoblasts were seeded at either 10,000 or 20,000 cells per well suspended in 100 µl of media, corresponding to 50% and 100% confluency, respectively. The plates were placed in the incubator for 12 hrs and the wells were washed twice with PBS to remove non-adherent cells before removal of the inner annulus. Each well was
visualized and digitized with an inverted microscope (JENA, Sedival, Seiler Instrument, St Louis, MO). For each well of the 12-well plate, a mosaic of 192 images was spliced together in a 12 X16 grid with the aid of a automated motorized stage and LabView software (National Instruments, Austin, TX) at an objective magnification of 10x to get a high-resolution image of the entire well for analysis. Scion Image software (Scion, Frederick, MD) was utilized for analysis.

The images taken 12 hrs after cell seeding were considered day 0 of the migration experiment. Day 0 images seeded at 50% confluency were examined in their entirety for isolated cells not in contact with other cells and used to determine the surface area covered by a marrow-derived osteoblast as a quantitative measure of cell spreading. Isolated cells in a 12X16 mosaic were magnified and manually outlined. Scion Image was utilized to quantitate the area covered by each cell. For each formulation, at least 150 cells were analyzed. The mean of the distribution was used for comparison between groups.

Cell migration was assessed during the 3-day period after the removal of the inner annulus by digitizing each well every 24 hrs. The surface area covered by the marrow-derived osteoblasts was determined by morphometric analysis. The cell region was visually selected by drawing a line along the migration front. The surface area of the megacolONY covering the hydrogel was measured.

After the 3-day period, the total cell number remaining on the films was assessed by measuring the total DNA content of each well with a PicoGreen assay as previously described.
5.2.6 Statistical Analysis

Means were analyzed with analysis of variance (ANOVA) using Tukey’s HSD (highly statistically different) multiple comparison test where an alpha level of 0.05 was defined as significant. Reported measurements are represented as means ± standard deviation unless otherwise stated (n=4 for EWC, adhesion, and migration experiments, n>5 for contact angle, and n>150 for area covered by a marrow-derived osteoblast).

5.3 Results

5.3.1 Hydrogel Characterization

Three hydrogels, CP750, CP2000, and CP5000, based on a mPEG block number average molecular weight of 800, 1960 and 5190, respectively, of the P(PF-co-EG) block copolymer were fabricated in the form of thin films. The equilibrium water content and contact angle of each copolymer is presented in Table 5-1. One-way Tukey’s HSD analysis revealed a significant (p<0.05) increase in equilibrium water content with each increase in mPEG molecular weight of the P(PF-co-EG) copolymer. Additionally, analysis of contact angle by copolymer revealed a significant difference between CP750 and either CP2000 or CP5000. However, addition of covalently linked peptide did not significantly affect either the equilibrium water content of the resulting hydrogel or the static contact angle.
5.3.2 Cell Cryopreservation and Culture

The cryopreserved marrow-derived osteoblasts were thawed and their viability was assessed at greater than 85%. Cells were plated in T-75 flasks with the osteogenic supplements for a length of culture from 5 to 7 days before covering 75-85% of the culture plate. The cells were then lifted and utilized in the adhesion and migration experiments.

Table 5-1. Physical characterization of P(PF-co-EG) hydrogels via equilibrium water content (EWC) and static contact angle as a function of copolymer composition and covalently linked RGDS concentration. Reported values represent means ± standard deviation for n=4 (EWC) and n>5 (contact angle).

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>RGDS Concentration (nmol/ml)</th>
<th>EWC (Percent)</th>
<th>Contact Angle (Theta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP750</td>
<td>0</td>
<td>39.6±1.6</td>
<td>40.3±9.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>42.2±1.0</td>
<td>38.0±5.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>43.5±1.5</td>
<td>40.8±5.3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>43.6±1.0</td>
<td>29.3±3.6</td>
</tr>
<tr>
<td>CP2000</td>
<td>0</td>
<td>52.5±0.3</td>
<td>20.6±2.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>52.5±1.1</td>
<td>17.5±3.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>53.3±0.8</td>
<td>15.7±6.5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>53.3±0.3</td>
<td>14.8±4.8</td>
</tr>
<tr>
<td>CP5000</td>
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<td>60.1±0.2</td>
<td>19.5±1.9</td>
</tr>
<tr>
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<td>62.2±1.4</td>
<td>18.2±4.3</td>
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</tr>
<tr>
<td></td>
<td>1000</td>
<td>63.0±2.2</td>
<td>17.3±6.9</td>
</tr>
</tbody>
</table>

5.3.3 Cell Adhesion Assay

Marrow-derived osteoblasts cultured in the osteogenic supplements were visualized 12 hrs after seeding onto the hydrogel (Figure 5-1). Qualitatively, cells cultured on CP750 were more spread when compared to CP2000 and CP5000. Quantitatively, the
adhesion of marrow-derived osteoblasts to hydrogels with covalently linked RGDS was dependent on both the mPEG block length of the P(PF-co-EG) and the covalently linked RGDS concentration (Figure 5-2). For hydrogels synthesized with CP750, the covalently linked RGDS concentration did not have a significant effect on cell adhesion. However, for hydrogels synthesized with CP2000 and CP5000, an increase in covalently linked RGDS concentration resulted in a significant increase in cell adhesion.

The specificity of marrow-derived osteoblast adhesion to covalently linked RGDS was validated by two controls: (1) hydrogels synthesized with a scrambled control peptide RDGS (last column of Figures 2 and 3) and (2) cells incubated in media with 5 mM GRGD prior to seeding onto hydrogels (Figure 5-3). The scrambled peptide (RDGS) showed decreased cell adhesion when compared to the cell adhesive (RGDS) peptide sequence. Comparison of the adhesion of cells incubated with GRGD peptide with hydrogels with covalently linked RGDS or the scrambled peptide RDGS showed no significant differences. Soluble GRGD (Figure 5-3) inhibited cell adhesion on the hydrogels that were otherwise marrow-derived osteoblast adhesive with similar cell adhesion to that on hydrogels synthesized without any covalently linked peptide.

The area covered by a single cell was measured by video microscopy for hydrogels synthesized with CP2000. The cell size distributions for covalently linked RGDS concentrations of 10, 50, 100, and 1000 nmol/ml are shown in Figure 5-4 with mean ± standard error of the mean cell areas of 270 ± 10, 490 ± 30, 1150 ± 30, and 1320 ± 40 μm², respectively. There was a significant difference between each concentration and its preceding peptide concentration (p<0.05).
Figure 5-1. Representative digitized images of marrow-derived osteoblasts 12 hrs after seeding on unmodified hydrogels (a) CP750, (b) CP2000, and (c) CP5000, and on modified hydrogels with 1000 nmol/ml RGDS (d) CP750, (e) CP2000, and (f) CP5000. Arrows indicate cells that have attached and are well spread. Scale bar represents 50 μm.
Figure 5-2. Marrow-derived osteoblast adhesion to hydrogels based on (□) CP750, (□) CP2000, and (□) CP5000 as a function of covalently linked RGDS concentration or the scrambled RDGS control. Cell numbers were normalized to the initial seeding density of 40,000 cells. Error bars represent means ± standard deviation for n=4. The symbol (†) indicates a statistical significance with CP750 of the same peptide concentration. The symbol (§) indicates a statistical significance with immediately lower peptide concentration. The symbol (††) indicates a statistical significant with CP2000. The symbol (*) indicates a statistical significance between the RGDS peptide and the scrambled control.
Figure 5-3. Marrow-derived osteoblast adhesion to hydrogels based on (□) CP750, (□) CP2000, and (□) CP5000 as a function of covalently linked RGDS concentration or the scrambled RDGS control. Cells were suspended in media containing 5mM GRGD for 30 minutes prior to seeding. Cell numbers were normalized to initial seeding density of 40,000 cells. Error bars represent means ± standard deviation for n=4.
Figure 5-4. Distribution of surface area covered by an adhered marrow-derived osteoblast 12 hrs after seeding on hydrogels synthesized with CP2000 copolymer and covalently linked RGDS peptide in concentrations of (a) 10, (b) 50, (c) 100, and (d) 1000 nmol/ml for n>150. There is a statistical significance with each increase in concentration of peptide.
5.3.4 Megacolony Migration Assay

The megacolony migration assay was conducted using hydrogels synthesized from CP2000 with covalently linked RGDS concentrations of 100 and 1000 nmol/ml and seeded with cells at either 50 or 100% confluence, that is either 10,000 or 20,000 cells, respectively. Visual inspection of the marrow-derived osteoblasts showed an increasingly diffuse layer of cells at the periphery of the megacolony with increasing culture time (Figure 5-5). During the 3-day period of the migration experiment, there was a significant increase in the surface area coverage for each of the examined formulations (Figure 5-6). One-way Tukey's HSD analysis of surface coverage with RGDS concentration revealed a significant decrease in surface area coverage with the higher concentration of RGDS when comparing hydrogels seeded at the same seeding density. One-way analysis of cell seeding density revealed a significant decrease in surface area coverage for hydrogels seeded at 50% when compared to 100% confluency.

The total number of cells adhered to the hydrogels after the 3-day migration assay was determined and normalized to the initial seeding density (Figure 5-7). Hydrogels with 10 and 50 nmol/ml covalently linked RGDS had less than 50% of the initially seeded cells adhered after 3 days. For hydrogels with 100 and 1000 nmol/ml covalently linked RGDS, a significant increase in the total number of cells was measured for formulations seeded at 50% confluency when compared to those seeded at confluency. No differences in cell number after three days in culture were found when comparing hydrogels with RGDS concentrations of 100 and 1000 nmol/ml.
Figure 5-5. Partial image of the leftmost edge of a representative mosaic of a marrow-derived osteoblast megacolony cultured on a P(PF-co-EG) hydrogel consisting of CP2000 with covalently linked RGDS at a concentration of 1000 nmol/ml (a) after removal of the inner annulus and (b) after 3 days in culture at the same location on the same hydrogel. Black line represents the outer-most cell layer utilized for quantification. Filled arrows represent the initial boundary of the megacolony. Open arrows represent the boundary of the megacolony after 3 days. Scale bar represents 50 μm.
Figure 5-6. Culture surface area of migrating marrow-derived osteoblasts seeded at 100% confluency of the inner annulus on hydrogels synthesized with covalently linked RGDS peptide sequence at a concentration of (□) 100 and (■) 1000 nmol/ml and seeded at 50% confluency on hydrogels synthesized with covalently linked RGDS peptide sequence at a concentration of (Δ) 100 and (▲) 1000 nmol/ml as a function of culture time. Error bars represent means ± standard deviation for n=4. The symbol (‡) indicates a statistical significance with the lower seeding density of the same peptide concentration. The symbol (§) indicates a statistical significance with the lower peptide concentration of the same seeding density. The symbol (†) indicates the first time point where a statistically significant increase in surface area coverage occurred compared to the initial surface area coverage.
Figure 5-7. Proliferation of marrow-derived osteoblasts after 3 days in culture seeded at either (◼) 50% or (□) 100% confluency on hydrogels synthesized with covalently linked RGDS peptide sequence at a concentration of 10, 50, 100, and 1000 nmol/ml. Cell numbers were normalized to the initial seeding density. Error bars represent means ± standard deviation for n=4. The symbol (‡) indicates a statistical significance with the lower seeding density of the same peptide concentration.
5.4 Discussion

The objective of this study was to synthesize a BIM based on the biodegradable copolymer P(PF-co-EG) by reducing non-specific cell adhesion and imparting specific receptor-mediated adhesion via covalent attachment of a model RGDS peptide sequence. Furthermore, the ability of adhered marrow-derived osteoblasts to migrate over the BIM surface was quantified in an attempt to elucidate the effect of the concentration of covalently attached peptide sequence on the migration characteristics of marrow-derived osteoblasts.

The effect of the osteogenic supplements utilized in this study to differentiate marrow stromal cells to the osteoblastic lineage has been well documented [Aubin, 1999; Bruder et al., 1997; Bruder et al., 1998; Caplan et al., 2001; Cooper et al., 2001]. Additionally, cryopreservation of marrow-derived osteoblasts has been shown to have no effect on their growth or osteogenic differentiation [Bruder et al., 1997; Cooper et al., 2001].

The first step of synthesizing a material capable of specific receptor-ligand adhesion was to reduce non-specific cell adhesion. Under physiological conditions in the presence of serum or extracellular matrix proteins, cell-substrate adhesion is mediated by the adsorption of proteins to the substrate. Biomaterials incorporating hydrophilic polymers including PEG on their surface have been shown to decrease protein adsorption, and thus diminish non-specific cell adhesion [Amiji et al., 1993; Bearinger et al., 1997; Jeon et al., 1991; Tziampazis et al., 2000].

For unmodified hydrogels, both CP2000 and CP5000 showed minimal cell adhesion with a lack of cell spreading. However, 12 hrs after cell seeding, CP750 showed a
significantly greater number of adhered cells when compared to CP2000 and CP5000. The hydrophobicity of CP750 could be one possible explanation for the increased cell adhesion compared to the other hydrogels. These hydrogels have the greatest contact angle and lowest equilibrium water content compared to CP2000 and CP5000. An increase in the hydrophobicity of the biomaterial affects the quantity, conformation, and type of protein adsorbed onto the biomaterial surface, ultimately leading to an increase in cell adhesion for the range studied [Tziampazis et al., 2000; van Wachem et al., 1987].

Increasing the concentration of covalently linked RGDS did not significantly affect the number of attached marrow-derived osteoblasts to CP750. The spacer arm of the RGDS (~3400 MW), was much longer than the mPEG block length of the P(PF-co-EG), (~750 MW). The highly crosslinked network could have prevented the mobility of the PEG spacer arm of the RGDS peptide [Jeon et al., 1991]. Unable to move about freely, the peptide could be trapped within the CP750 hydrogel and unavailable for cell recognition at the surface of the hydrogel.

There was a RGDS concentration dependent adhesion of marrow-derived osteoblasts on both CP2000 and CP5000 hydrogels. A RGDS concentration of 100 nmol/ml was sufficient for significant cell adhesion to CP2000 and CP5000, where 84 ± 17% and 74 ± 14% of the initial seeded cells attached to the hydrogels after 12 hrs, respectively. However, for a RGDS concentration of 1000 nmol/ml, there was a significantly greater number of marrow-derived osteoblasts adhered on CP2000, 91 ± 10%, compared to CP5000, 62 ± 8%. The mPEG block length of CP5000 was greater than the spacer arm of the peptide. The longer mPEG block length could mask the peptides available to the
marrow-derived osteoblasts which reduces the effective RGDS concentration available for the cells [Shin et al., 2002].

Soluble GRGD prevented the adhesion of marrow-derived osteoblast to hydrogels with modified RGDS by blocking the integrin receptors of the marrow-derived osteoblasts. A 5 mM concentration of soluble GRGD has been previously shown to be sufficient for the prevention of adhesion of many cell types [Dee et al., 1998; Shin et al., 2002]. Both the soluble GRGD and the scrambled covalently linked control provided evidence that the adhesion of marrow-derived osteoblast onto the modified hydrogels was through a specific integrin-peptide interaction.

The projected area of the adhered marrow-derived osteoblasts was dependent on the RGDS concentration. This area has been shown to be an indicator of the adhesivity of the cells to the biomaterial [Rezania et al., 1999a; Webb et al., 2000], where cell spreading is increased due to greater number of receptor-ligand bonds. By increasing the linked RGDS concentration, more RGD ligands are available for the cells, allowing for more cell receptor-ligand bonds, ultimately resulting in greater cell adhesivity to the hydrogel. Marrow-derived osteoblast spreading and adhesivity was greater on hydrogels with increasing covalently linked RGDS concentrations.

Cell spreading and adhesivity has also been shown to be an indicator of migration speed [Hynes, 1992; Webb et al., 2000]. For this study, marrow-derived osteoblasts cultured on hydrogels modified with 100 nmol/ml were less spread, less adhesive, but covered more surface area while those cultured on hydrogels modified with 1000 nmol/ml were more spread, more adhesive, and covered less surface area after 3 days.
The megacolony migration assay, a cell-population migration assay, cannot easily isolate the effects of cell migration and proliferation [Barrandon et al., 1987; Lee et al., 1995]. After removal of the inner annulus, the marrow-derived osteoblasts can concurrently migrate and proliferate. For all formulations reported in the migration experiments, the periphery of the megacolony was diffuse with cells, suggesting that the increase in surface area coverage was not due to proliferation alone. Additionally, a change in the concentration of the linked RGDS peptide, from 100 to 1000 nmol/ml, did not affect the proliferation rates of the marrow stromal cells while decreasing the cell surface area coverage. This is supporting evidence that the increase in cell surface area coverage was not entirely due to cell proliferation and was dependent on cell migration.

For the RGDS concentrations of 100 and 1000 nmol/ml, the seeding density had a significant effect on the marrow-derived osteoblast surface coverage, which was found to be lower for hydrogels seeded at 50% confluency. Marrow-derived osteoblasts seeded at 50% confluency onto the hydrogels could have been migrating locally within the megacolony without an appreciable difference in the overall surface area coverage. For these hydrogels with linked RGDS concentration of 100 and 1000 nmol/ml seeded at 50% confluency, doubling times of approximately 3 days was found, similar to doubling times for canine [Kadiyala et al., 1997] and human [Bruder et al., 1997] marrow-derived osteoblasts cultured on tissue culture polystyrene.

Marrow-derived osteoblasts seeded at confluency onto hydrogels did not proliferate significantly. The large size of the megacolony could contribute to a lack of a significant increase from the initially seeded cell number. The increase in surface area coverage due
to the proliferation of the outermost cell layer does not significantly contribute to the surface area coverage of the megacolony.

The megacolony migration assay does have limitations. Only a limited covalently linked RGDS concentration was utilized in this study. Cell size and proliferation was measured on hydrogels synthesized with CP2000 and covalently linked RGDS concentrations of 10, 50, 100, and 1000 nmol/ml. However, marrow-derived osteoblast migration on hydrogels with covalently linked RGDS peptide in concentrations of 10 and 50 nmol/ml could not be quantitated. For low RDGS concentrations of 10 and 50 nmol/ml, the low adhesivity of the marrow-derived osteoblasts to the hydrogels prevented a concentrated cell population. Hydrogels synthesized with 10 and 50 nmol/ml RGDS had less than 20% and 50%, respectively, of the initially seeded cells present after 3 days in culture. These hydrogels did not provide a suitable surface for long-term proliferation of the marrow-stromal osteoblasts studied.

P(PF-co-EG) hydrogels with a covalently linked peptide may provide a BIM for modulating cell function and guiding tissue formation. The ability to form porous scaffolds of these materials in situ renders them very versatile for use in tissue engineering. Their potential use as carriers for biologically active molecules may facilitate the localized delivery of tissue morphogens to induce a tissue regeneration cascade.
5.5 Conclusions

Marrow-derived osteoblast adhesion was dependent on the mPEG block length of the P(PF-co-EG) with mPEG block lengths of 2000 and 5000 reducing nearly all non-specific cell adhesion. An RGDS concentration of 100 nmol/ml covalently linked to P(PF-co-EG) based hydrogels with an mPEG block length of 2000 was sufficient to promote cell adhesion of 84 ± 17 % of the initial seeded cells after 12 hrs. A megacolony migration assay conducted on P(PF-co-EG) hydrogels with an mPEG block length of 2000 over a 3-day period showed that the covalently linked RGDS concentration did not have an effect on the proliferation rates of marrow-derived osteoblasts. However, the migration rate was greater on hydrogels synthesized with an RGDS concentration of 100 nmol/ml when compared to those synthesized with 1000 nmol/ml.
CHAPTER 6*

Quantification of Ligand Surface Concentration of Bulk-Modified Biomimetic Hydrogels

Chapter Abstract

This study describes a method for the quantification of active ligand surface concentration for bulk-modified hydrogels. Two poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) block copolymers were synthesized with terminal poly(ethylene glycol) (PEG) chains of number average molecular weight 1960 and 5190 g/mol. Hydrogels were synthesized with bulk-modified biotin as a model ligand, making use of a PEG spacer arm with a molecular weight of 3400 g/mol. Bulk concentration of biotin was calculated from the initial concentration of biotin, sol fraction, equilibrium water content, and relative incorporation of the polymers to the hydrogel. Surface concentration of biotin bulk-modified hydrogels was quantified with an enzyme linked immunosorbent assay using mouse monoclonal anti-biotin antibody (IgG), horseradish peroxidase-conjugated anti-mouse IgG, and a chemiluminescent substrate. The larger size of the IgG relative to the mesh size of the hydrogels allowed for the quantification of the active biotin at the surface of the hydrogels. Luminescent imaging was used to qualitatively show the isolation of the horseradish peroxidase-conjugated antibodies to

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the surface of the bulk-modified hydrogel. The active biotin ligands at the surface of hydrogels synthesized with terminal PEG chains of 1960 g/mol were at the top 7.2 nm while for those synthesized with terminal PEG chains of 5190 g/mol were at the top 4.4 nm of the bulk-modified hydrogel. The relationship between bulk ligand concentration and the active ligand concentration at the surface was dependent on the hydrogel composition. The relative magnitude of the PEG spacer arm of the ligand compared to the PEG block length of the copolymer affected the surface availability of the ligand. The results suggest that steric hindrances caused by mobile PEG chains of the copolymer of molecular weight greater than that of the PEG spacer arm contributed to the decreased surface concentration of ligand. This work relates the bulk concentration of a ligand to its surface concentration, an important parameter for the adhesion, migration, and function of anchorage dependent cells.

6.1 Introduction

Synthetic hydrogels, which are usually biologically inert materials, may be modified with protein fragments or peptide sequences to modulate cell behavior at the biomaterial-tissue interface [Behravesh et al., In Press-b; Drumheller et al., 1994; Gobin et al., 2002; Healy et al., 1999; Hern et al., 1998; Rezania et al., 1999a; Shin et al., 2002]. To promote a specific cell-receptor interaction, these hydrogels may be modified in the bulk with cell adhesion specific peptide ligands. However, to date, the surface concentrations
of these bulk-modified hydrogels have only been estimated [Elbert et al., 2001; Hern et al., 1998; Shin et al., 2002].

Surface ligand concentration has been accurately quantified in two-dimensional systems through radioactive labeling combined with surface modification. The cell adhesion peptide ligand surface concentration has been shown to be an integral parameter in cell adhesion [DiMilla et al., 1993; Massia et al., 1991; Neff et al., 1998] and migration [DiMilla et al., 1993; Kouvroukoglou et al., 2000], but these studies have been conducted on surface-modified model materials.

For biomimetic hydrogels, the availability of covalently linked ligands to cells was found dependent on the length of a spacer arm, usually of poly(ethylene glycol) (PEG), linking the ligand to the bulk material [Deguchi et al., 1999; Hern et al., 1998]. In addition, the concentration of the bulk-modified cell adhesive peptides has been shown to affect cell adhesion, migration, and function [Behravesh et al., In Press-b; Gobin et al., 2002; Rowley et al., 2002; Shin et al., 2002].

Poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) block copolymer hydrogels have shown promise for tissue engineering applications [Behravesh et al., 2002a; Behravesh et al., 2002b; Suggs et al., 1999b]. These hydrogels are based on the biodegradable copolymer P(PF-co-EG) having terminal PEG blocks. The PEG block length of P(PF-co-EG)-based hydrogels modified with a peptide ligand, relative to the PEG spacer arm linking the peptide to the hydrogel backbone, was shown to affect osteoblastic cell adhesion [Behravesh et al., In Press-b]. The availability of the peptide to the cell for P(PF-co-EG)-based hydrogels and other fumarate-based hydrogels [Shin et
al., 2002] was found to be dependent of the ratio of the PEG block length of the copolymer to the PEG spacer arm of the peptide ligand.

In this study, biotin was covalently linked to P(PF-co-EG) hydrogels and utilized as a model ligand. Biotin was selected because of its small size similar to that of cell adhesive peptides, its lack of functional groups that can participate in the crosslinking reaction of the hydrogel, and the availability of IgG antibodies for its quantification using an enzyme linked immunosorbent assay (ELISA). The overall objective was to quantify the surface concentration of biotin covalently linked to the bulk of P(PF-co-EG) hydrogels with a PEG spacer arm. In addition, by synthesizing two P(PF-co-EG) hydrogels with varying PEG block lengths, the effect of the PEG block length of the copolymer relative to the PEG spacer of the biotin ligand on the availability of that ligand at the surface of the hydrogel was assessed.

6.2 Materials and Methods

6.2.1 Polymer Synthesis

Two block copolymers of poly(propylene fumarate) (PPF) and poly(ethylene glycol) (PEG) were synthesized with terminal methoxy poly(ethylene glycol) (mPEG) blocks as previously described [Behravesh et al., 2002b]. These copolymers, CP2000 and CP5000, were synthesized from mPEG having a number average molecular weight of 1960 and 5190 g/mol, respectively, and with, on average, 1.7 and 1.5 terminal mPEG blocks,
respectively, with a middle PPF block of number average molecular weight of approximately 1400 g/mol [Behravesh et al., 2002b].

Poly(ethylene glycol)-diacrylate (PEG-DA) having a nominal molecular weight of 700 g/mol and a number average molecular weight of 860 g/mol calculated by nuclear magnetic resonance (NMR) spectroscopy, was purchased from Aldrich (Milwaukee, WI).

Acryloyl-poly(ethylene glycol)-biotin (Ac-PEG-Biotin) was synthesized using established methods [Behravesh et al., In Press-b; Hern et al., 1998]. Briefly, Ac-PEG-Biotin was synthesized from acryloyl-poly(ethylene glycol) N-hydroxysuccinimide (Ac-PEG-NHS) (Shearwater, Huntsville, AL) of poly(ethylene glycol) (PEG) nominal molecular weight of 3400 and 5-(2-oxo-hexahydro-thieno [3,4-d]imidazol-6-yl)-pentanoic acid {2-[2-(2-amino-ethoxy)-diethoxy]-ethyl}-amide (Pierce, Rockford, IL) (Figure 6-1), a water-soluble biotin containing molecule with a single primary amine. Ac-PEG-NHS and the biotin containing molecule were dissolved separately in 5 mM sodium bicarbonate buffer, pH 8.2, at a final concentration of 75 mg/ml and 1 mg/ml, respectively. A molar ratio of 2:1 Ac-PEG-NHS to the biotin containing molecule was utilized to ensure a complete reaction of the biotin. The Ac-PEG-NHS solution was added drop-wise to the biotin containing solution with vigorous stirring and allowed to react for 2 hrs. Low molecular weight by-products and sodium bicarbonate were removed via dialysis using a molecular weight cut-off dialysis membrane of 2000 g/mol (Spectrum Laboratories, Rancho Dominguez, CA) with 5 double distilled deionized water (DDW) changes over the course of 2 days. The Ac-PEG-Biotin was lyophilized for 48 hours and stored below -4°C until use.
Figure 6-1. Water-soluble biotin molecule with one primary amine utilized in the synthesis of acryloyl-poly(ethylene glycol)-biotin.

6.2.2 Hydrogel Synthesis

P(PF-co-EG) was mixed with PEG-DA on a 1:1 wt% basis and dissolved with vigorous mixing in DDW in a final polymer concentration of 66 wt%. Subsequently, Ac-PEG-biotin was added to the polymer solution at 5 different concentrations of 0, 10, 50, 100, 500, 1000 nmol/cm$^3$ in their hydrogel’s final swollen state based on the equilibrium water content of the non-biotinylated unmodified hydrogel. The polymer solution was centrifuged to remove small bubbles trapped within the aqueous solution. The redox initiation system for the crosslinking reaction consisted of an oxidizer, ammonium persulfate (APS) (Acros, Pittsburgh, PA), combined with a reducer, ascorbic acid (AH) (Aldrich). APS and AH were added sequentially to the polymer each at a final concentration of 0.01 M. The solution was cast either between two glass plates with a 0.5 mm spacer (thin film) or a cylindrical mold 2 mm in height and 4 mm in diameter (cylinder) and allowed to crosslink overnight at 37°C. The synthesis scheme and final structure of the hydrogel are illustrated in Figure 6-2.
Figure 6-2. Reaction scheme for and structure of crosslinked hydrogel with covalently linked biotin.
6.2.3 Hydrogel Characterization

Thin films of hydrogels were swollen in DDW for at least 24 hours and were cut either into thin disks 10 mm in diameter for equilibrium polymer volume fraction calculations or into dog-bone shapes using a stainless steel cutter for tensile testing.

The equilibrium polymer volume fraction of the swollen hydrogel \( \nu_{2,s} \) was calculated as previously described [Peppas et al., 1986; Shung et al., In Press]:

\[
\nu_{2,s} = \frac{V_p}{V_{g,s}}
\]

The volume of the equilibrium swollen hydrogel, \( V_{g,s} \), was measured after weighing samples, equilibrium swollen in DDW, in air \( (W_{a,s}) \) and in cyclohexane (Fisher, Pittsburgh, PA) \( (W_{n,s}) \) using a hanging pan balance as follows:

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

where \( \rho_n \) is the density of the non-solvent cyclohexane \( (\rho_n=0.774 \text{ g/cm}^3) \). Samples were air dried overnight, vacuum dried for 24 hours, and then weighed \( (W_{a,d}) \). The volume of the dried hydrogel, \( V_p \), was calculated as follows:

\[
V_p = \frac{W_{a,d}}{\rho_p}
\]
where $\rho_p$ is the density of the polymer (1.15 g/cm$^3$) [Shung et al., In Press].

For tensile testing, glass slides were glued using cyanoacrylate to the ends of the swollen dog-bone shaped hydrogels to provide a stable gripping surface. The samples prepared for tensile testing were 33 mm in length, 6 mm wide, and approximately 1 mm in thickness at the narrowest point. The exact dimensions were measured prior to testing using a digital caliper. An Instron 5500 series tabletop load frame (Instron Corporation, Canton, MA) was utilized at a crosshead speed of 10 mm/min to test each sample in tension until failure. The stress and strain data were recorded and the Young’s modulus ($E$) was determined from the slope of the initial linear portion of the stress/strain curve. The molecular weight between crosslinks ($M_C$) was estimated by assuming an affine deformation [Sperling, 1992]:

$$M_C = \frac{3\rho_p RT}{E} \nu_{2,s}^{1/3}$$

where $R$ is the gas constant and $T$ is the temperature. The mesh size, $\xi$, was calculated by assuming that the active chains of the hydrogel network are composed of PEG, originating from PEG-DA, as previously described [Peppas et al., 1986; Temenoff et al., 2002]:

$$\xi = \nu_{2,s}^{-1/3} \left( 3 \cdot C_n \cdot \frac{M_C}{M_t} \cdot i^2 \right)^{1/2}$$
assuming $C_n = 4.0$, the characteristic ratio, $M_r = 44 \text{ g/mol}$, the molecular weight of the PEG repeat unit, and $l = 1.47 \text{ Å}$, the weighted average of the backbone bond lengths for PEG.

### 6.2.4 Gravimetry

Thin film samples were cut with a cork borer to a diameter of 5 mm. Each sample was air dried overnight and vacuum dried for 24 hrs to remove all residual water. Samples were weighed individually ($W_d$) and swollen in excess phosphate buffered saline (PBS) for 48 hours with 3 changes on PBS. The surface water of the samples was then removed with the aid of weight paper and the sample weights were measured ($W_s$). Each sample was then vacuum dried and reweighed ($W_{rd}$). The sol fraction was calculated as:

$$\text{Sol fraction} = \frac{(W_d - W_{rd})}{W_d}$$

The equilibrium water content was measured as follows:

$$\text{Equilibrium water content (\%)} = \frac{(W_s - W_{rd})}{W_s} \times 100\%$$

### 6.2.5 Nuclear magnetic resonance spectroscopy

Hydrogel thin films were dried in air overnight and in vacuum for at least 48 hours to remove initial water. Approximately 100 mg of the dried hydrogels were swollen in 2 ml
of deuterium oxide. Proton NMR measurements were performed with a 400 MHz spectrometer (Bruker, Switzerland). Integration of the proton peaks from the end methoxy group of the terminal mPEG blocks of P(PF-co-EG) were compared to the proton peaks of the acrylate group of the PEG-DA to determine the ratio of P(PF-co-EG) to PEG-DA in the sol fraction. The chemical shift of the acrylate protons of the Ac-PEG-biotin and PEG-DA were assumed to be similar. This ratio was converted to a weight basis using the molecular weights and the number of protons of the functional groups per mole of the acrylated (PEG-DA and Ac-PEG-Biotin) and methoxy containing (P(PF-co-EG)) polymer. The fraction of acrylated polymers incorporated was calculated by the following equations:

\[
\text{Acrylate incorporated (\%) = } \frac{\phi - \theta}{\phi} \cdot 100\%
\]

\[
\theta = \text{Sol fraction} \left(1 + \frac{w_p}{w_{Ac}}\right)^{-1} \cdot \tau
\]

where \(\phi\) represents the weight of the initial acrylated polymers, \(\theta\) is the weight of the acrylated polymers in the sol fraction, \(\tau\) is the total weight of the polymers and \(w_p / w_{Ac}\) is the weight ratio of P(PF-co-EG) to the acrylated polymers, PEG-DA and Ac-PEG-Biotin.

6.2.6 Chemiluminescence Assays

A NightOWL imaging system (Perkin-Elmer, Shelton, CO) was utilized to visualize the localization of the biotin bound horseradish peroxidase (HRP) conjugated anti-biotin IgG in a chemiluminescent substrate. Hydrogel cylinders (3 mm in height and 5 mm in
diameter) and thin films (8 mm in diameter) were swollen in PBS for at least 48 hrs. Next, 1 ml of mouse anti-biotin (Molecular Probes, Eugene, OR) in dilution 1:5000 was added to each well. The hydrogels were incubated for 1 h at 37°C with gentle agitation (60 rpm) and subsequently rinsed with PBS three times. As a secondary antibody, goat anti-mouse IgG conjugated with HRP (Pierce) (~2.5 HRP per antibody) in 1:2500 dilutions was used. The hydrogels were incubated for 1 h at 37°C and then washed as described above. A vertical slice from the cylinder was cut (~1 mm in thickness) using a razor blade from the middle of the cylinder. The thin films and vertical slices were then placed flat in a 24-well tissue culture plate. The chemiluminescent ELISA substrate (Pierce) (300 μl) was added to each well and the plate was briefly shaken. After a 2 min incubation in the substrate, the samples were placed in the camera dark box. Images were acquired with a supercooled CCD NightOWL camera and processed using the WinLight32 software (Perkin-Elmer) with an exposure time of 1 min at a medium resolution (2 x 2 pixel binning).

A luminometer was utilized to quantitate the active concentration of biotin on the hydrogel’s surface. Equilibrium swollen hydrogels were cut using a cork borer to 1.8 cm diameter disks, placed in 12-well plate, incubated in PBS with 0.05% bovine serum albumin for 1 h, and washed three times with PBS. As before, 1 ml of mouse anti-biotin in dilution 1:10000 was added to each well. The hydrogels were incubated for 1 h at 37°C and subsequently rinsed with PBS three times. The secondary antibody, HPR-conjugated goat anti-mouse, was used in 1:5000 dilutions. The hydrogels were incubated for 1 h at 37°C and then washed three times.
The samples having a diameter of 1.8 cm were then cut using a cork borer to disks of 2.5 mm in diameter, excluding the outer perimeter of the initial disk. They were then placed in a luminometer test tube to which 50 µl of PBS were added. The chemiluminescent substrate (Pierce) (150 µl) was added to each tube vortexed for 30 s and placed into the luminometer. After 60 s, the light output from each sample was integrated for 10 s after a 2 s delay by the luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). The signal was recorded as relative luminescence units and calibrated to the secondary antibody of a known concentration where the molecular weight of the HRP linked secondary antibody was 250,000 g/mol.

6.2.7 Antibody Surface Coverage

The percent surface area covered by the secondary IgG antibody on the hydrogel was estimated by

\[
\text{Surface area coverage (\%)} = \pi \cdot r_{ab}^2 \cdot S \cdot N_{AV} \cdot 100\%
\]

where, \( r_{ab} \) is the hydrodynamic radius of the IgG antibody (54 Å [Franssen et al., 1999]), \( N_{AV} \) is Avogadro’s number, and \( S \) (mol/cm\(^2\)) is the measured surface concentration of primary antibody, assuming a one to one stoichiometry between the primary and secondary antibody.
6.2.8 Statistical analysis

All data are reported as means ± standard deviation, and compared using analysis of variance (ANOVA). A student's t-test was used to analyze differences between groups. The p-values are reported for groups tested with statistical significance. For calculated values, standard deviations are reported with propagation of error [Taylor, 1982].

6.3 Results

6.3.1 Hydrogel Characterization

Hydrogels were characterized via swelling and mechanical testing. The polymer volume fraction, Young’s modulus, molecular weight between crosslinks, and mesh size are tabulated (Table 6-1). The two formulations, CP2000 and CP5000 had similar polymer volume fractions of 0.40 ± 0.00 and 0.39 ± 0.02, respectively, but different tensile moduli of 6.35 ± 0.62 and 2.99 ± 0.55 MPa, molecular weight between crosslinks of 1000 ± 100 and 2070 ± 40 g/mol, and mesh sizes of 33 ± 2 and 48 ± 1 Å, respectively.

Table 6-1. Characterization of P(PF-co-EG) hydrogels from equilibrium swelling experiments and mechanical testing. Reported values are means ± standard deviation for n=5.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Polymer volume fraction</th>
<th>Young’s modulus (MPa)</th>
<th>Molecular weight between crosslinks (g/mol)</th>
<th>Mesh size (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP2000</td>
<td>0.40 ± 0.00</td>
<td>6.35 ± 0.62</td>
<td>1000 ± 100</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>CP5000</td>
<td>0.39 ± 0.02</td>
<td>2.99 ± 0.55</td>
<td>2070 ± 40</td>
<td>48 ± 1</td>
</tr>
</tbody>
</table>
6.3.2 Gravimetry

Hydrogels were synthesized without significant differences in equilibrium water content and sol fraction with increasing biotin concentrations for either CP2000 or CP5000 (Table 6-2). One-way student’s t-test analysis revealed a significant (p<0.05) increase in equilibrium water content with an increase in the mPEG molecular weight of the P(PF-co-EG) copolymer. Equilibrium water content was between 55.2 ± 0.4 and 57.9 ± 0.3 % for CP2000 and between 58.9 ± 0.5 and 60.1 ± 0.1 % for CP5000. The sol fraction was less than 0.05 for all formulations.

Table 6-2. Characterization of biomimetic P(PF-co-EG) hydrogels bulk-modified with biotin. Reported values are means ± standard deviation for n=5.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Theoretical Biotin Concentration (nmol/cm³)</th>
<th>Ac-PEG-Biotin / P(PF-co-EG) (mg/g)</th>
<th>Equilibrium Water Content (%)</th>
<th>Sol Fraction</th>
<th>Actual Biotin Concentration (nmol/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP2000</td>
<td>0</td>
<td>0</td>
<td>57.7 ± 0.4</td>
<td>0.043 ± 0.006</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.32</td>
<td>55.2 ± 0.4</td>
<td>0.024 ± 0.014</td>
<td>10.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.6</td>
<td>56.1 ± 1.0</td>
<td>0.015 ± 0.013</td>
<td>55.2 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.2</td>
<td>56.3 ± 0.6</td>
<td>0.015 ± 0.016</td>
<td>108.6 ± 9.4</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>16</td>
<td>56.2 ± 0.3</td>
<td>0.028 ± 0.010</td>
<td>532.5 ± 28.8</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>32</td>
<td>58.0 ± 0.3</td>
<td>0.037 ± 0.008</td>
<td>1002.3 ± 45.9</td>
</tr>
<tr>
<td>CP5000</td>
<td>0</td>
<td>0</td>
<td>59.3 ± 0.1</td>
<td>0.021 ± 0.008</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.37</td>
<td>58.9 ± 0.5</td>
<td>0.027 ± 0.013</td>
<td>11.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.85</td>
<td>59.4 ± 0.4</td>
<td>0.031 ± 0.014</td>
<td>57.9 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>3.7</td>
<td>60.0 ± 0.4</td>
<td>0.043 ± 0.008</td>
<td>114.9 ± 11.1</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>18.5</td>
<td>60.0 ± 0.5</td>
<td>0.018 ± 0.008</td>
<td>564.2 ± 55.5</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>37</td>
<td>60.1 ± 0.1</td>
<td>0.026 ± 0.011</td>
<td>1090.0 ± 145.0</td>
</tr>
</tbody>
</table>

6.3.3 Nuclear Magnetic Resonance

Proton NMR of the sol fraction was acquired to quantitate the fraction of acrylated polymers incorporated to the hydrogel (Figure 6-3). The ratio of the acrylate to methoxy
functional group protons was 1.7 to 1 for CP2000 and 0.8 to 1 for CP5000 hydrogels. The incorporation of the acrylated polymers was determined to be greater than 99% by weight for both copolymers utilized (between 99.1% and 99.7% for CP2000 and 99.7 and 99.9% for CP5000). The relative incorporation of the acrylated polymers was utilized for the calculation of the bulk biotin concentration covalently linked to the hydrogel (Table 6-2). The theoretical bulk biotin concentrations were within the standard deviation of their calculated values.

![NMR Spectrum](image)

**Figure 6-3.** Representative $^1$H nuclear magnetic resonance spectrum of the sol fraction of the unmodified CP2000 hydrogel in D$_2$O. The symbols (†, §, †') represent the acrylate protons of the poly(ethylene glycol)-diacrylate. The symbol (‡) represents the methoxy end group protons of the poly(propylene fumarate-co-ethylene glycol).
6.3.4 Chemiluminescence Assays

The luminescent imaging of the cross-section of the hydrogel cylinders, Figure 6-4 a-d, showed that the luminescence was localized on the surface of the biotin-modified hydrogels and was absent in unmodified hydrogels. In addition, luminescence on the surface of the biotin-modified hydrogels was well distributed for the biotin-modified hydrogels (Figure 6-4 e-h). No luminescence was visualized from unmodified hydrogels, Figure 6-4 a, b, e, and f.

The chemiluminescent ELISA revealed that the active surface biotin concentration was dependent on the biotin bulk concentration (Figure 6-5). The biotin surface concentration of the bulk-modified hydrogels (~ 50, 100, and 500 nmol/cm³) was significantly greater for CP2000 when compared to CP5000. Bulk concentrations up to 500 nmol/cm³ were utilized to fit a linear curve for CP2000. All points were utilized for curve fitting of CP5000. The slope of the curves was 0.72 and 0.44, with R² values of 0.984 and 0.995 for CP2000 and CP5000, respectively.

6.3.5 Surface Area Coverage

The surface area of the hydrogels covered by the anti-biotin IgGs was calculated based on a hydrodynamic radius of 54 Å (Table 6-3). The surface area covered by the antibodies reached a maximum of 23.5 ± 7.6 % for CP2000 and 26.9 ± 9.8 % for CP5000 for the highest bulk concentration examined. The Surface area coverage for CP2000 hydrogels modified at two highest concentrations of biotin, 500 and 1000 nmol/cm³, were similar, 20.8 ± 4.9 and 26.9 ± 9.8%, respectively.
Figure 6-4. Representative images of cross-sectional slices (Images a-d) of cylinders 5 mm in diameter and 3 mm in height using the NightOWL imaging system under normal light (a and c) and luminescence (b and d), and top surfaces of hydrogels (Images e-h) under normal light (e and g) and luminescence (f and h). Images a-b and e-f are of control hydrogels while images c-d and g-h are of hydrogels modified with biotin at a concentration of 1000 nmol/cm$^3$. 
Figure 6-5. Surface concentration of biotin for bulk-modified CP2000 (□) and CP5000 (■) hydrogels as a function of bulk concentration of biotin. The dashed line represents the linear fit of the data. The symbol (*) represents a statistical difference between hydrogel formulations at the same bulk biotin concentration. Error bars represent standard deviations for n=4.

Table 6-3. Characterization of surface coverage of IgG antibodies on hydrogels.

<table>
<thead>
<tr>
<th>Theoretical Bulk Concentration (nmol/cm³)</th>
<th>CP2000</th>
<th></th>
<th>CP5000</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface Concentration (fmol/cm²)</td>
<td>IgG Surface Coverage (%)</td>
<td>Surface Concentration (fmol/cm²)</td>
<td>IgG Surface Coverage (%)</td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10</td>
<td>3 ± 3</td>
<td>0.2 ± 0.1</td>
<td>5 ± 7</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>50</td>
<td>72 ± 39</td>
<td>4.0 ± 2.2</td>
<td>16 ± 17</td>
<td>0.9 ± 1.0</td>
</tr>
<tr>
<td>100</td>
<td>99 ± 54</td>
<td>5.5 ± 3.0</td>
<td>54 ± 26</td>
<td>3.0 ± 1.4</td>
</tr>
<tr>
<td>500</td>
<td>377 ± 88</td>
<td>20.8 ± 4.9</td>
<td>220 ± 116</td>
<td>12.1 ± 6.4</td>
</tr>
<tr>
<td>1000</td>
<td>426 ± 139</td>
<td>23.5 ± 7.6</td>
<td>488 ± 178</td>
<td>26.9 ± 9.8</td>
</tr>
</tbody>
</table>
6.4 Discussion

The objective of this study was to develop a method of quantifying the surface concentration of biotin ligands covalently linked to the bulk of hydrogels. Biotin modification was conducted with chemistries similar to those utilized for bulk modification of hydrogels with peptides [Behravesh et al., In Press-b; Hern et al., 1998]. Two P(PF-co-EG) block copolymers with varying terminal PEG block lengths, CP2000 and CP5000, were utilized to vary the ratio of the PEG block length of the copolymer to the PEG block length of the spacer arm linking the biotin to the backbone of the hydrogel. The relationship between the biotin bulk concentration and the active biotin concentration available at the surface of the hydrogels was assessed by an ELISA chemiluminescent technique.

Hydrogels based on P(PF-co-EG) were synthesized without a significant difference in sol fraction. For both formulations, an amount greater than 95% by weight of the initial polymers including P(PF-co-EG), PEG-DA, and Ac-PEG-biotin was incorporated into the hydrogel. In addition, the \(^1\)H NMR of the sol fraction showed that greater than 99% of the initial acrylated polymers and approximately 96% of the initial P(PF-co-EG) copolymers were incorporated to the hydrogel. This difference in the incorporation of acrylate (PEG-DA and Ac-PEG-biotin) to fumarate (P(PF-co-EG) copolymer) illustrated the greater reactivity of the acrylate bond in comparison to the fumarate bond known in the literature [Greenley, 1989]. Because of this difference in reactivity of the functional groups, quantification of the relative amount of polymers incorporated into the hydrogel was necessary to accurately calculate the bulk concentration of covalently linked biotin.
The success of the quantification of surface concentration was dependent on the absence of penetration of the IgG antibodies into the bulk of the hydrogel. The mesh size of \(33 \pm 2\) and \(48 \pm 1\) Å, for CP2000 and CP5000, respectively, was less than the hydrodynamic diameter of an IgG, 108 Å. For these highly crosslinked hydrogels studied, the antibodies were not able to penetrate into the bulk of the hydrogel and were only able to react with the antigens available at the surface of the hydrogels. The absence of luminescence in the middle of the cross-sectional slice of the biotin-modified hydrogels corroborated these results. In addition, a low background luminescence for unmodified hydrogel samples, similar to those not exposed to the secondary antibody, supported this assumption, as entrapped secondary antibodies within the bulk of the hydrogel would contribute to luminescence upon exposure to the chemiluminescent substrate. These results suggested that the antibodies could only interact with biotin at the surface of the hydrogel.

From the luminometer results, linear trends of biotin surface concentration with increasing bulk concentration for the two formulations studied were evident; however, the surface concentration of biotin for CP2000 at the highest concentration tested, 1000 nmol/cm\(^3\), did not follow the linear trend and was much lower than expected. The surface coverage of the IgG molecules provided an explanation. With an IgG hydrodynamic diameter of 108 Å, the surface coverage was found reach a maximum at \(20.8 \pm 4.9\) and \(23.5 \pm 7.6\) % for CP2000 modified with biotin bulk concentrations of 500 and 1000 nmol/cm\(^3\), respectively. This plateau of a maximum measurable surface concentration has been previously examined for ligand modified surfaces and has been shown to be dependent on the size of the molecules used for the measurement [Herron et
al., 1995; Huang et al., 2002]. For example, 1 to 2 pmol/cm² was found to be the maximum surface concentration of IgG antibodies to form a fully packed surface layer [Herron et al., 1995].

For this study, a secondary IgG with, on average 2.5 linked HRP molecules was utilized. Because of the larger size of the HRP-conjugated antibody in comparison to an IgG, the maximum surface concentration of the secondary antibody is expected to be less than 1 pmol/cm². In fact, the maximum measurable surface concentration in this study was found to be less than 500 fmol/cm². For CP2000, there was no significant increase in the measured surface concentration comparing hydrogels modified with 500 and 1000 nmol/cm³ biotin (377 ± 88 and 426 ± 139 fmol/cm², respectively). Because of the size of the IgG secondary antibody, a maximum measurable surface concentration was reached, where, because of steric hindrance, IgG molecules already bound to the surface blocked access for other IgG molecules.

Another potential factor reducing the measured surface concentration for high ligand densities is the multivalency of a IgG antibodies. At low biotin surface concentrations, the ligands are sufficiently spaced from each other to limit the binding of biotin ligands to IgG in a 1 to 1 ratio. At higher concentrations, however, two biotin ligands can potentially interact with one anti-biotin IgG. This could be enhanced by the flexibility of the biotin ligand because of the long PEG spacer arm. For these reasons, the highest concentration of biotin for CP2000 was not included in the linear fit.

Previously, for P(PF-co-EG)-based hydrogels modified in the bulk with RGD cell adhesive peptides, a bulk concentration of 100 nmol/cm³ was found to promote cell adhesion for both CP2000 and CP5000, while a concentration of 1000 nmol/cm³ was
found to enhance cell spreading [Behravesh et al., In Press-b]. Utilizing the linear fit of the bulk vs. surface concentration data, this study showed that bulk concentrations of 100 and 1000 nmol/cm³ correspond to surface concentrations of 72 and 720 fmol/cm², respectively for CP2000, and 44 and 440 fmol/cm², respectively for CP5000. These values correlate well to studies conducted by modifying glass surfaces with known peptide concentrations which have shown that concentrations as low as 10 fmol/cm² were sufficient for cell adhesion (formation of stress fibers) [Massia et al., 1991]. For surfaces modified with cell adhesive ligands using mobile PEG chains, a concentration of 60 fmol/cm² was required for cell adhesion, while a concentration of 470 fmol/cm² was required for well-spread cells [Neff et al., 1998; Neff et al., 1999].

In addition, these results suggested that the availability of the ligand at the surface was dependent on the structure of the hydrogel, specifically, the ratio of the PEG terminal chains to the PEG spacer arm of the biotin ligand. When the PEG chains of the copolymer are larger than the PEG spacer arm, steric hindrance may prohibit the access of the antibody to the ligand at the surface of the hydrogel. This may explain the differences in the surface accessibility of the biotin, mentioned above between the tested formulations, CP2000 and CP5000.

Estimations found in the literature of the surface concentration of bulk-modified hydrogels assumed that the top 10 nm composed the surface of the hydrogel [Hern et al., 1998; Shin et al., 2002]. In this study, the active biotin ligand surface concentration corresponded to a surface layer of 7.2 nm for CP2000 and 4.4 nm for CP5000, calculated from the slope of the fitted line (Figure 6-5). Although this technique did provide a relationship between bulk ligand concentration and its surface concentration, no general
relationship that could be applied to all hydrogels was established. The availability of the ligand at the surface of the hydrogel to the antibodies was not only dependent on the ligand itself but also on the ratio between the PEG block lengths of the copolymer and the PEG spacer of the ligand and cannot be extrapolated to other hydrogel networks. However, the method of quantification could be applied to other bulk-modified hydrogel systems provided that the hydrogel's mesh size is much smaller than the IgG hydrodynamic diameter.

6.5 Conclusion

A new method was developed to relate the bulk ligand concentration of biomimetic hydrogels to the ligand concentration available at the surface. Biotin was used as a model ligand to quantify the surface concentration of bulk-modified hydrogels. Two P(PF-co-EG) block copolymers were used to test the effect of the terminal PEG chain length in comparison to the PEG spacer length of the biotin on the availability of the biotin at the surface of the hydrogel. CP2000 hydrogels (PEG number average molecular weight of 1960 g/mol and PEG spacer arm molecular weight of 3400 g/mol) had greater concentration of biotin available at the surface when compared to CP5000 hydrogels (PEG block number average molecular weight of 5190 g/mol and PEG spacer arm molecular weight of 3400 g/mol). An effective penetration depth of IgG to active biotin ligands was found to be 7.2 nm for CP2000 and 4.4 nm for CP5000. A maximum
measurable biotin surface concentration was reached because of the size of the secondary IgG antibody causing steric hindrance or the antibody’s multivalency.
CHAPTER 7*

Synthesis of In Situ Crosslinkable Biodegradable Macroporous Hydrogels Based on Poly(Propylene Fumarate-co-Ethylene Glycol)

Chapter Abstract

This study describes a synthesis method of biodegradable macroporous hydrogels suitable as in situ crosslinkable biomaterials. Macroporous hydrogels were based on poly(propylene fumarate-co-ethylene glycol) and prepared via coupled free radical and pore formation reactions. Crosslinking was initiated by a pair of redox initiators, ammonium persulfate and L-ascorbic acid. Pores were formed by the reaction between L-ascorbic acid and sodium bicarbonate, a basic component, which evolved carbon dioxide. Sol fraction of the hydrogels was varied from 0.06 ± 0.01 to 0.64 ± 0.01. A stereological approach was used to analyze the morphological properties of the macroporous hydrogels by relating the morphological properties of thin sections to the original three-dimensional macroporous hydrogel. Prepared macroporous hydrogels had porosities between 0.43 ± 0.08 to 0.84 ± 0.02 and surface area densities between 55 ± 3 to 108 ± 7 cm⁻¹. Sodium bicarbonate concentration had the greatest effect on both the porosity and surface area density. The effect of copolymer formulation on the porosity and surface area density was insignificant. From thin sections of the macroporous

hydrogels, the profile size distributions were determined as an estimate of the pore size
distribution. Two formulations synthesized with varying L-ascorbic acid concentration
of 0.05 M and 0.1 M had median profile sizes of 50-100 μm and 150-200 μm, respectively. This novel synthesis method allows for the *in situ* crosslinking of
biodegradable macroporous hydrogels with morphological properties suitable for
consideration as an injectable tissue engineering scaffold.

7.1 Introduction

Many methods have been developed for the fabrication of macroporous scaffolds for
tissue engineering applications. Prefabricated porous scaffolds with controlled
morphological parameters such as porosity and pore size distribution have been prepared
by a number of techniques including fiber bonding, particulate leaching, freeze-drying,
phase separation, gas foaming, and 3-dimensional printing [Chen et al., 2000;
Lewandrowski et al., 1999; Mooney et al., 1996; Nam et al., 2000; Park et al., 1998;
Robinson et al., 1995; Widmer et al., 1998]. However, prefabricated scaffolds are not
suitable for irregularly shaped or hard to reach defects.

*In situ* crosslinkable polymers addressing this issue have been synthesized for
orthopaedic applications [Anseth et al., 1997; Peter et al., 1999b; Peter et al., 1997c].
Poly(propylene fumarate) (PPF), an amorphous unsaturated polymer, has been
extensively studied as an injectable biomaterial for bone tissue engineering utilizing
particulate leaching to achieve a porous scaffold [Peter et al., 1998]. Furthermore, the
copolymerization of PPF with poly(ethylene glycol) (PEG) resulted in poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), a water soluble copolymer developed and crosslinked for thin film cardiovascular applications [Suggs et al., 1998a]. Both in vitro and in vivo, P(PF-co-EG) based hydrogels were shown to be biodegradable during a 12-week period [Suggs et al., 1998b]. Thin P(PF-co-EG) hydrogel films have allowed for intimate contact with surrounding tissue and have shown minimal fibrous encapsulation in vivo [Jo et al., 2000; Suggs et al., 1998b]. Using an in vivo rat cage implant system, P(PF-co-EG) hydrogels exhibited an exudate leukocyte concentration comparable to that of the negative control. Moreover, P(PF-co-EG) has the potential to be covalently functionalized with integrin binding peptides making P(PF-co-EG) based hydrogels potentially cell adhesive [Jo et al., 2000]. The described properties of P(PF-co-EG) hydrogels are favorable for their application as a biocompatible scaffolding material. However, existing scaffold fabrication techniques do not have the potential for in situ formation of macroporous hydrogels.

Fabrication of a macroporous hydrogel suitable for in situ formation will require concurrent pore formation and crosslinking reactions to entrap the resulting macropores within the hydrogel. Chemical methods of free-radical polymerization have been utilized for in situ crosslinkable polymers [Temenoff et al., 2000]. Redox initiation systems have been an effective method of generating free-radicals under mild, aqueous conditions [Allcock et al., 1981; Sarac, 1999]. The water-soluble initiator of a persulfate salt (S\textsubscript{2}O\textsubscript{8}\textsuperscript{2-}), when mixed with a reducer yields a sulfate (SO\textsubscript{4}\textsuperscript{2-}) and a radical ion (SO\textsubscript{4}\textsuperscript{2+}). Additionally, bicarbonate salts have been utilized to form carbon dioxide, acting as a porogen [Chen et al., 2000; Lewandrowski et al., 1999; Nam et al., 1999]. The redox pair
of persulfate and ascorbic acid have been studied for polymerization reactions with the rate of polymerization dependent, among other parameters, on the initiator concentration [Sarac, 1999]. In redox initiation systems, the amount of oxidizer and reducer play an important role in the initiation rate and ultimately on the final material properties.

Control over morphological parameters of porosity, surface area density, and pore size distribution can play an important role in the biomaterial performance affecting degradation rates and interaction with host tissue. For example, the surface area density has been shown to play an important role in vitro and in vivo degradation characteristics of poly(\(\alpha\)-hydroxy acid) foams [Lu et al., 2000a; Lu et al., 2000b]. The surface area density of bioerodable macroporous polymers can have an effect on the degradation and release rates of solutes [Zygourakis et al., 1996]. The pore size distribution and surface area density of poly(\(\alpha\)-hydroxy acid) foams can have an affect on the bone formation in calvarial defects [Robinson et al., 1995; Wang et al., 1999].

In this study, a combination of a water soluble redox initiation reaction involving ammonium persulfate and L-ascorbic acid, and a CO\(_2\) forming reaction involving sodium bicarbonate, were used to produce three-dimensional macroporous biodegradable P(PF-co-EG) copolymer hydrogels. A full factorial experimental design was utilized to assess the main effects of PEG block length of the P(PF-co-EG) copolymer, ammonium persulfate concentration, and sodium bicarbonate content on the sol fraction, porosity, and surface area density of the resulting macroporous hydrogels. Additionally, the effects of L-ascorbic acid concentration and sodium bicarbonate content on the sol fraction, porosity, and surface area density of the produced macroporous hydrogels were studied.
7.2 Materials and Methods

7.2.1 Poly(Propylene Fumarate-co-Ethylene Glycol) Synthesis

Poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) was synthesized as previously described [Behravesh et al., 2002b]. Briefly, using a three neck round bottom flask with overhead mechanical stirring, bis-(2-hydroxypropyl) fumarate was synthesized under a nitrogen atmosphere by a reaction of diethyl fumarate (Acros, Pittsburgh, PA) and propylene glycol (Fisher, Pittsburgh, PA) at 160°C with zinc chloride (Sigma, St. Louis, MO) and hydroquinone (Sigma). The product was then transesterified under vacuum (<1 mmHg) at 150°C to form poly(propylene fumarate) (PPF). Progression of the molecular weight of the PPF was monitored every hour by gel permeation chromatography (GPC) using polystyrene standards until a number average molecular weight of 2050 (polydispersity index = 1.8) was achieved. P(PF-co-EG) was then synthesized by the transesterification reaction of methoxy poly(ethylene glycol) (mPEG) (Aldrich Chemical, Milwaukee, WI) and PPF. Two copolymers, namely CP750 and CP2000, were synthesized by reacting PPF with mPEG of nominal molecular weights of 750 and 2000, respectively, on a mPEG:PPF molar ratio of 2:1. The number average molecular weight of mPEG with nominal molecular weight 750 and 2000 was measured by nuclear magnetic resonance spectroscopy as 800 and 1960, respectively [Behravesh et al., 2002b]. The extent of the transesterification was monitored by GPC.

The copolymer was then dissolved in methylene chloride (Fisher) and purified by precipitation in ethyl ether (Fisher). Copolymers were either waxy or solid at room
temperature. Ethyl ether was decanted for a waxy copolymer and any remaining solvent was removed by rotoevaporation. A powdery copolymer was isolated by filtration. Copolymers were subsequently dried under vacuum at room temperature to remove any residual organic solvent.

7.2.2 Scaffold Fabrication

The hydrogel formulation was composed of the block copolymer, P(PF-co-EG), and poly(ethylene glycol) diacrylate (PEG-DA) (Aldrich Chemical) in a 1:1 weight ratio. PEG-DA of nominal molecular weight 700 was utilized. The polymers were dissolved in double distilled water on a 33% by volume basis. The redox initiation system for the crosslinking reaction consisted of an oxidizer, ammonium persulfate (APS) (Acros), combined with a reducer, L-ascorbic acid (AH) (Aldrich Chemical). The reaction of sodium bicarbonate (SB) (Aldrich Chemical) and acid produced the porogen, carbon dioxide. SB, APS, and AH were added sequentially. Each component was well mixed with the polymer solution before the subsequent addition of the next component.

A factorial experimental design was employed to assess the effects of mPEG block length of P(PF-co-EG), APS concentration, and SB content [Neter et al., 1996]. High and low values for the three parameters are listed in Table 7-1a. The formulation compositions are numerated in Table 7-1b. In addition to the factorial design, the effect of AH concentration was assessed independent of copolymer formulation and APS concentration. AH concentration was varied at two levels, 0.05 M and 0.1 M, while sodium bicarbonate concentration was varied at three levels: 30, 60, and 90 mg/ml.
Table 7-1a. High and low levels for three parameters tested in two-level factorial design.

<table>
<thead>
<tr>
<th>Level</th>
<th>mPEG Block Molecular Weight</th>
<th>APS (moles/l)</th>
<th>SB Content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (+)</td>
<td>2000</td>
<td>0.25</td>
<td>90</td>
</tr>
<tr>
<td>Low (-)</td>
<td>750</td>
<td>0.05</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 7-1b. Combination of experimental variables in a two-level factorial design.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>mPEG Block Molecular Weight</th>
<th>APS (moles/l)</th>
<th>SB Content (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

7.2.3 Temperature Rise

The temperature rise of the exothermic crosslinking reaction was measured during crosslinking of P(PF-co-EG) and PEG-DA in a Teflon chamber (inner dimensions: 1 cm diameter, 1.5 cm height; outer dimensions: 2 cm diameter, 2 cm height) held at a constant temperature of 37°C in a water bath [Pascual et al., 1996]. The polymer solution containing P(PF-co-EG), PEG-DA, APS, and SB was prepared at room temperature. At time zero, the AH solution was added with rapid mixing to the aqueous prepolymer solution and quickly poured into the Teflon chamber. A thermocouple (Omega, KMTSS-
032U-6, Stamford, CT) was inserted to half the depth of the mold, and temperature readings were recorded every 15 s.

7.2.4 Sol Fraction

Macroporous hydrogels were prepared by crosslinking 7.5 ml of the polymer solution overnight in a 2.5 cm inner diameter glass vial. The hydrogels were dried overnight in a laminar flow hood and dried further under vacuum (<1 mmHg) for 24 hours. They were then cut longitudinally into quarters and their weights were measured \( W_d \). Samples were then hydrated for 48 hours in double distilled water with gentle agitation. The hydrated hydrogels were subsequently redried by the previously described procedure and their weights were measured \( W_{rd} \). Sol fraction of the macroporous hydrogels was determined by the following equation:

\[
\text{Sol Fraction} = \frac{W_d - W_{rd}}{W_d}
\]

7.2.5 Scanning Electron Microscopy

Dried porous hydrogels prepared as described in the previous experimental section were briefly placed in liquid nitrogen and subsequently fractured. Samples were then coated with gold using a sputter coater (CRC-100, Plasma Sciences, Lorton, VA) at a current of 100mA for 60 s. Micrographs were obtained with a SEM (JOEL JSM-5300 Boston, MA) using 15 keV accelerating potential.
7.2.6 Morphometric Analysis

The morphology of the swollen macroporous hydrogels was determined by a stereological approach, a method that relates the two-dimensional thin section to the three-dimensional structure. Macroporous hydrogels were swollen in pH 7.4 phosphate buffered saline (PBS) (GibcoBRL, NY) at 37°C for 48 hours to mimic the *in vivo* environment. Hydrated hydrogels were removed from the PBS and three horizontal segments (~0.5 cm) were cut at different vertical heights for each sample by using a scalpel with a #10 blade. The hydrogel segments were then placed in an embedding medium for frozen tissue specimens (VWR, Willard, OH). Samples were embedded under vacuum (~300 mmHg) for 24 hours to improve penetration of the embedding media.

Sections of each segment for all formulations were prepared by a Carl Zeiss HM 505 Cryostat Microtome to a thickness of 50 μm and dried on a slide warmer at 60°C. The sections were stained with 0.1% w/v toluidine blue-O (Aldrich Chemical) in PBS for 10 s and then washed twice in double distilled water. A Nikon R600 microscope was used for visualization. Analyses of the digitized images (magnification 4X corresponding to an image size of 5.5 mm by 3.66 mm) were performed using Scion Image v. 1.62. At least, 10 images were analyzed for each formulation.

The method of morphometric analysis is illustrated in Figure 7-1. A representative initial digitized image is shown in Figure 7-1a. Thresholding of the image was performed using a HSL (hue, saturation, lightness) thresholding plug-in from Image Processing Tool Kit v 3.0 (Reindeer Games, Inc., 1999) (Figure 7-1b). Subsequently, irregularities in the staining procedure were corrected manually by comparison to the
original section. With the aid of Scion Image analysis software, the fraction of pore area was measured by taking the ratio of white pixels to the total number of pixels. The thresholded images were then outlined (Figure 7-1c). The fraction of pore perimeter was measured by taking the ratio of black to total pixels. Unbiased estimators of porosity ($\varepsilon$) and surface area density ($S_v$) were calculated as follows [Zygourakis, 1993]:

$$\varepsilon = \frac{A_m}{A_p}$$

$$S_v = \frac{4}{\pi} \frac{B_m}{A_p}$$

where, within a sample field, $A_m$ is the cross-sectional area of the hydrogel macropores, $A_p$ is the total cross-sectional area, and $B_m$ is the perimeter or the boundary length of the pores as measured by the outlining procedure. The fraction $A_m / A_p$, a dimensionless unit, represents the ratio of pore area to the total area of each section. Similarly, the fraction $B_m / A_p$ represents the ratio of pore perimeter to total area of each section with dimension of inverse length. The thresholded image was then inverted and a watershed function was used to segment the connected pores (Figure 7-1d). From these segmented two-dimensional images, assuming a spherical pore shape, an estimate of the profile size distribution of the pores were measured by comparing each pore’s perimeter and area as follows:

$$D = 4 \frac{P_s}{P_p}$$
where \( D \) is the estimated pore profile diameter, \( p_a \) is the pore profile area, and \( p_p \) is the pore profile perimeter. Pores touching the edge of the image or less than 10 \( \mu \text{m} \) in diameter were not included in the analysis.

Figure 7-1. Stereological evaluation of macroporous hydrogels. (a) Representative thin section of porous hydrogel; (b) thresholded image with white denoting the pore space for determination of porosity; and (c) outlined image for determination of surface area density of swollen porous hydrogel. (d) For determination of profile size distribution, connected pores were segmented using a watershed function. Gray pores, those bordering image edges or less than 10 microns in diameter, were not included in the analysis. Scale bar represents 0.5 mm.
7.2.7 Statistical Analysis

Reported measurements for the full factorial experimental design are reported as the main effect ± standard error [Neter et al., 1996]. For the elucidation of the effect of AH and SB, Tukey's HSD multiple comparison test was utilized at an alpha level of 0.05 to test for significance between groups for sol fraction, porosity, and surface area density measurements. Reported measurements are represented as means ± standard deviation.

7.3 Results

7.3.1 Synthesis of Porous Hydrogels

Two copolymers, CP750 and CP2000, were synthesized by the transesterification reaction of PPF with mPEG of nominal molecular weight 750 and 2000, respectively. Gel permeation chromatography conferred number average molecular weights of 3600 (PI=1.4) for CP750 and 6730 (PI=2.2) for CP2000. CP750 was waxy while CP2000 was powdery at room temperature. Both copolymers were soluble in aqueous solution.

Porous hydrogels were synthesized with gelation occurring between 1 to 15 min following addition of AH. Gelation times were dependent on the APS concentration, with an increase in APS resulting in shorter gelation times. Moreover, there was a lack of a temperature rise above 37°C for all formulations.
7.3.2 Characterization of Porous Hydrogels

PBS was replaced with embedding medium for frozen tissue specimens with a negligible change in the dimensions of the swollen macroporous hydrogel. Utilizing the cryosectioning technique, the interconnected macroporous hydrogel was visualized with clear delineation of pore structure. The thresholding procedure was semi-quantitative, first performed by HSL thresholding procedure followed by manual correction of the irregularities in the staining procedure.

From the full factorial experiment, the effects of copolymer composition and the amounts of APS and SB on the sol fraction (Figure 7-2), porosity (Figure 7-3), and surface area density (Figure 7-4) of the resulting porous hydrogel were measured. Average values for sol fraction, porosity and surface area density were 0.4, 0.68, and 87 cm\(^{-1}\), respectively. The copolymer composition showed a minimal effect on the sol fraction and an insignificant effect on the porosity and surface area density. The sol fraction of the porous hydrogels showed a significant decrease with an increase in APS concentration. The most significant factor contributing to the porosity and surface area density of the macroporous hydrogels was the quantity of SB. The high and low values and their corresponding formulation of the sol fraction, porosity and surface area density are tabulated (Table 7-2).

<table>
<thead>
<tr>
<th></th>
<th>Sol Fraction</th>
<th>Porosity</th>
<th>Surface Area Density (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation</td>
<td>0.64 ± 0.01</td>
<td>0.78 ± 0.01</td>
<td>108 ± 7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Low</td>
<td>Mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formulation</td>
<td>0.06 ± 0.01</td>
<td>0.43 ± 0.08</td>
<td>75 ± 5</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 7-2. High and low measured values of sol fraction, porosity and surface area density from the full factorial experiment.
Figure 7-2. The main effect of the mPEG block molecular weight of P(PF-co-EG) copolymer, ammonium persulfate (APS) concentration, and sodium bicarbonate content on the sol fraction of porous hydrogels. For a particular parameter, a positive number indicates an increasing effect, while a negative number indicates a decreasing effect on the sol fraction as the value of the parameter was changed from a low level (-) to a high level (+) (See table 1a). Error bars represent the standard error of the effect (n>10).
Figure 7-3. The main effect of the mPEG block molecular weight of P(PF-co-EG) copolymer, ammonium persulfate (APS) concentration, and sodium bicarbonate content on the porosity of porous hydrogels. For a particular parameter, a positive number indicates an increasing effect, while a negative number indicates a decreasing effect on the porosity as the value of the parameter was changed from a low level (-) to a high level (+) (See table 1a). Error bars represent the standard error of the effect (n>10).
Figure 7-4. The main effect of the mPEG block molecular weight of P(PF-co-EG) copolymer, ammonium persulfate (APS) concentration, and sodium bicarbonate content on the surface area density of porous hydrogels. For a particular parameter, a positive number indicates an increasing effect, while a negative number indicates a decreasing effect on the surface area to volume ratio as the value of the parameter was changed from a low level (-) to a high level (+) (See table 1a). Error bars represent the standard error of the effect (n>10).

For formulations with varying AH concentrations, using CP2000 and a constant APS concentration of 0.25 M, sol fraction ranged from $0.28 \pm 0.01$ to $0.46 \pm 0.01$ (Figure 7-5). There was a significant increase of the sol fraction with increased AH concentration.
From the morphological analysis, an unbiased estimator of the porosity of the hydrogels was calculated and varied from approximately $0.66 \pm 0.03$ to $0.84 \pm 0.02$ (Figure 7-6). There was a significant increase in porosity with an increase in either AH or SB. Unbiased estimator of the surface area density ranged from approximately $55 \pm 3$ to $88 \pm 3 \text{ cm}^{-1}$ (Figure 7-7). SB had a significant effect on the surface area density for an AH concentration of 0.1 M as shown on the graph.

7.3.3 Scanning Electron Microscopy

SEMs were taken to show the morphology of the porous hydrogels in their dried state. Representative SEM showed multiple sites of interconnection (Figure 7-8).

7.3.4 Profile Size Distribution

In addition to the porosity and surface area density measurements, the profile size distribution for macroporous hydrogels synthesized with 60 mg/ml of sodium bicarbonate and an APS concentration of 0.25 M were analyzed. Profile size distributions were categorized into 50 μm increments (Figure 7-9). A median profile size of 50-100 μm and 150-200 μm was measured for an AH concentration of 0.05 and 0.1 M, respectively.
Figure 7-5. Variation of sol fraction of P(PF-co-EG) hydrogels as a function of sodium bicarbonate (SB) content for L-ascorbic acid (AH) in concentrations of (□) 0.1 M and (■) 0.05 M. Error bars represent standard deviations (n>10). The symbol (*) indicates a statistical significance between AH concentrations of the same SB concentration (p<0.05). The symbol (+) indicates a statistical significance with the immediately lower concentration of SB of the same AH concentration (p<0.05).
Figure 7-6. Variation of porosity of P(PF-co-EG) hydrogels as a function of sodium bicarbonate (SB) content for L-ascorbic acid (AH) in concentrations of (□) 0.1 M and (■) 0.05 M. Error bars represent standard deviations (n>10). The symbol (*) indicates a statistical significance between AH concentrations of the same SB concentration (p<0.05). The symbol (+) indicates a statistical significance with the immediately lower concentration of SB of the same AH concentration (p<0.05).
Figure 7-7. Variation of surface area density of P(PF-co-EG) hydrogels as a function of sodium bicarbonate (SB) content for L-ascorbic acid (AH) in concentrations of (□) 0.1 M and (■) 0.05 M. Error bars represent standard deviations (n>10). The symbol (*) indicates a statistical significance between AH concentrations of the same SB concentration (p<0.05). The symbol (+) indicates a statistical significance with the immediately lower concentration of SB of the same AH concentration (p<0.05).
Figure 7-8. Representative SEM of macroporous hydrogels with multiple sites of pore interconnection. Formulation consists of an ammonium persulfate concentration of 0.25 M, a sodium bicarbonate content of 60 mg/ml, and L-ascorbic acid concentration of 0.05 M.
Figure 7-9. Probability distribution of pore sizes for two formulations with an ammonium persulfate concentration of 0.25 M and a sodium bicarbonate amount of 60 mg/ml and an L-ascorbic acid concentration of (a) 0.05 M and (b) 0.1 M.
7.4 Discussion

The objective of this study was to describe a novel technique to synthesize biodegradable macroporous hydrogels with a potential for in situ crosslinking and towards this objective, investigate the effects of P(PF-co-EG) copolymer formulation, initiator concentration, and SB content on the morphological properties of the resulting macroporous hydrogels. Two P(PF-co-EG) copolymers with varying mPEG block lengths were synthesized. A water-soluble redox initiation system was employed for the crosslinking reaction. A CO₂ gas forming reaction produced pores necessary for the fabrication of a macroporous hydrogel that have the potential to be crosslinked in situ, entrapping macropores within the hydrogel.

The following scheme was proposed to describe the redox initiation and pore formation reactions:

\[
\begin{align*}
C_6H_8O_5 & \rightarrow H^+ + C_6H_7O_5^- \\
S_2O_8^{2-} + C_6H_7O_5^- & \rightarrow SO_4^{2-} + SO_4^{2-} + C_6H_7O_5^-
\end{align*}
\]

\[
\begin{align*}
HCO_3^- + H^+ & \rightarrow CO_2 + H_2O
\end{align*}
\]

The first reaction involved the dissolution of ascorbic acid. The second reaction of persulfate and ascorbic acid generated free radicals to initiate crosslinking [Allcock et al., 1981; Sarac, 1999]. The bicarbonate and proton reacted in the third reaction to form carbon dioxide, which constitutes the porous hydrogel structure. Crosslinking and pore formation reactions acted in parallel and were dependent on the AH concentration.
Although other charge transfer reactions were possible [Sarac, 1999], the above was a simple scheme for the initiation and pore formation reactions.

Both components of the redox initiation system had a significant effect on the sol fraction. The increase in APS concentration resulted in a decrease in the sol fraction, which could be due to increased free-radical production [Ariff et al., 1985; Bajpai et al., 1990]. Increasing the AH concentration led to an increase in sol fraction of the resulting macroporous hydrogel. APS has been traditionally paired with basic reducers such as N,N,N'N'-tetramethylethylenediamine [Chen et al., 2000]. Increasing the AH concentration relative to APS concentration resulted in a more acidic crosslinking formulation. The overall effect of pH on the rate of polymerization for persulfate-ascorbic acid initiation system was a decrease in the production of free-radicals resulting in lower polymerization rates [Ariff et al., 1985]. For example, gelation was inhibited entirely for a crosslinking formulation with twice the molar concentration of AH (0.1 M) compared to APS (0.05 M). This was a limiting factor of these initiators since acidic conditions are needed for the pore formation reaction. Only a limited AH range could be utilized for the formation of macroporous hydrogels.

For the two P(PF-co-EG) copolymers studied, the mPEG block length did not significantly affect the morphological properties of the resulting macroporous hydrogels (Figures 3-4). The PEG block length of PEG-modified biomaterials can play an important role in its interaction with host tissue. This fabrication technique allows for the synthesis of scaffolds of varying PEG block lengths without affecting the morphological properties of the macroporous hydrogel.
Porosities as great as $0.84 \pm 0.02$ were achieved with this technique which were sufficient for tissue engineering scaffolds [Thomson et al., 2000]. An increase in the SB content and AH concentration from 30 to 90 mg/ml and from 0.05 to 0.1 M, respectively, resulted in an increase in porosity from $0.66 \pm 0.03$ to $0.84 \pm 0.02$. The morphological parameters, porosity and surface area density could be related by the pore size distribution. Other fabrication techniques, such as particulate leaching utilizing soluble salts as a porogen, have the potential to alter the pore size distribution without significantly affecting the porosity. This technique, synthesizing macroporous hydrogels with concurrent crosslinking and pore formation, did not allow for a simple method of controlling the pore size. For example, higher porosity scaffolds had larger pore sizes.

The described stereological approach yielded unbiased estimators of porosity and surface area density. Utilizing a stereological approach, pore size distributions could theoretically be decomposed by several methods assuming a particular pore shape with no interconnections [Weibel, 1980]. Due to the highly interconnected nature and errors in the segmenting procedure the pore size distribution could not be quantitated (Figure 7-10). Therefore, the profile size distribution provided only an estimate of the pore size distribution, which could not be accurately calculated.

Alternatively, the surface area density alone could be utilized to estimate the theoretical pore size from geometry by assuming a perfectly packed foam structure with bubbles of a uniform size, with no interconnections as follows:

$$S_p = \frac{4\pi \cdot r^2 \cdot N^3}{(2r \cdot N)^3}$$
By solving for the radius of the theoretical carbon dioxide bubble, an estimated pore size could be calculated from the surface area density. Using this procedure, scaffolds with surface area densities of 50-100 cm\(^{-1}\) possess corresponding radii of 300-150 \(\mu\)m. The profile size distributions of the macroporous hydrogels illustrated in Figure 7-9 follow these results having median profile size distributions of 50-100 \(\mu\)m and 150-200 \(\mu\)m with significantly different surface area densities, 84 \(\pm\) 3 cm\(^{-1}\) and 66 \(\pm\) 3 cm\(^{-1}\), respectively.

Superporous hydrogels based on acrylamide monomer have been previously synthesized. These non-degradable superporous hydrogels were designed for their fast swelling characteristics and developed as gastric retentive drug-delivery vehicles [Chen et al., 2000; Dorkoosh et al., 2001; Gemeinhart et al., 2001]. In this study, macroporous hydrogels were based on P(PF-co-EG), a biodegradable, biocompatible material suitable as an injectable biomaterial [Suggs et al., 1998b; Suggs et al., 1999b]. Other techniques have been developed for prefabricated as well as \textit{in situ} formed macroporous constructs; however, no fabrication technique to date allows for the \textit{in situ} crosslinking of biodegradable macroporous hydrogels. The fabrication technique described in this study produced porous hydrogels with morphologies suitable for consideration as \textit{in situ} crosslinkable biomaterials for use as a tissue engineering scaffold.

Additionally, hydrogels crosslinked with free-radical polymerization have been previously modified with short oligopeptide sequences found in extracellular matrix proteins [Hern et al., 1998]. These short peptide chains have been shown to mediate adhesion, migration, and proliferation for specific anchorage dependent cells [Dee et al., 1996; Han et al., 1997; Hern et al., 1998; Rezania et al., 1999b]. Modification of the
macroporous hydrogel with these peptide sequences may provide a biomimetic scaffold for guided tissue regeneration.

Figure 7-10. A representative section for assessment of profile size distribution. Pores included in the profile size distribution analysis are black. Arrows indicate faulty segmentation of connected pores. Formulation consists of an ammonium persulfate concentration of 0.25 M, a sodium bicarbonate content of 60 mg/ml, and L-ascorbic acid concentration of 0.05 M.

7.5 Conclusion

A fabrication method of interconnected macroporous hydrogels with P(PF-co-EG) was described. Macropores were introduced by the formation of carbon dioxide during crosslinking. The morphological characteristics of the macroporous hydrogels were determined by stereological methods. Sol fraction, porosity and surface area density of the P(PF-co-EG) macroporous hydrogels were modulated by adjustment of the reactant
concentrations of the free-radical initiation and pore formation reactions. SEM of dry macroporous hydrogels suggested an interconnected pore structure within the tested range of parameters. These macroporous hydrogels synthesized with a water soluble initiation system for the crosslinking reaction have potential applications as *in situ* crosslinkable biomaterials.
CHAPTER 8*

Evaluation of the In Vitro Degradation of Macroporous Hydrogels Using Gravimetry, Confined Compression Testing, and Microcomputed Tomography

Chapter Abstract

This study investigated the in vitro degradation characteristics of macroporous hydrogels based on poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)). Four formulations were fabricated to test the effect of porosity and crosslinking density on the degradation of the resulting macroporous hydrogels. Macroporosity was introduced by the addition of sodium bicarbonate and ascorbic acid, the precursors of the carbon dioxide porogen, in the initiation system for the hydrogel crosslinking. Macroporous hydrogels with porosities of 0.80 ± 0.03 and 0.89 ± 0.03 were synthesized by the addition of sodium bicarbonate of concentration of 40 and 80 mg/ml and ascorbic acid of concentration of 0.05 and 0.1 moles/l, respectively. Poly(ethylene glycol)-diacrylate (PEG-DA) was utilized as a crosslinker. The molecular weight between crosslinks had a significant effect on weight loss after twelve weeks, where samples with $M_C$ of 1880 ± 320 synthesized with a P(PF-co-EG):PEG-DA ratio of 3:1 had a significantly greater mass loss due to degradation than those with $M_C$ of 1000 ± 100 synthesized with a P(PF-co-EG):PEG-DA ratio of 1:1. In contrast, porosity played a minimal role in determining

* This chapter is from: Behravesh, Timmer, Lemoine, Liebschner, and Mikos (In Press): Biomacromolecules.
the weight loss. Mechanical testing of the hydrogels under confined compression showed a decrease in compressive modulus over the degradation time for all formulations. In addition, an increase in hydrogel equilibrium water content and pore wall thickness was observed with degradation time, whereas the hydrogel porosity and surface area density remained invariant. The results from microcomputed tomography corroborated with the rest of the measurements and indicated a bulk degradation mechanism of the macroporous hydrogels.

8.1 Introduction

Porous biodegradable polymer scaffolds find many tissue engineering applications [Behravesh et al., 1999]. Porosity and pore size distribution have been shown to be important factors for vascularization [Mikos et al., 1993] and guided tissue regeneration [Yaszemski et al., 1995]. The degradation characteristics of porous scaffolds fabricated from hydrophobic polymers such as poly(α-hydroxy esters) have been well characterized [Lu et al., 2000a; Lu et al., 2000b]. Among other parameters, scaffold porosity has been shown to have a significant effect on the degradation both in vitro and in vivo [Lu et al., 2000a; Lu et al., 2000b].

Quantification of the morphology of macroporous constructs as tissue engineering scaffolds has been limited to porosity and surface area density measurements mainly by mercury intrusion porosimetry and stereology [Behravesh et al., 2002a; Issa et al., 1998; León y León, 1998]. Microcomputed tomography (μCT), a technique previously
employed to examine the structure of trabecular bone [Ulrich et al., 1999] has been recently used to characterize the morphology of porous polymer scaffolds [Zeltinger et al., 2001]. This technique allows for the reconstruction of the three-dimensional structure of macroporous solids and the measurement of additional material properties including the pore wall thickness and pore edge length which determine their potential applications.

Poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), a highly unsaturated in situ crosslinkable hydrophilic block copolymer with degradable ester linkages, was synthesized to macroporous hydrogel scaffolds with porosities dependent on the sodium bicarbonate (SB) and ascorbic acid (AH) concentrations of the initiation system [Behravesh et al., 2002a]. These hydrogels incorporated poly(ethylene glycol)-diacrylate (PEG-DA) as a crosslinker [Shung et al., In Press].

The purpose of this study was to elucidate the effect of porosity and molecular weight between crosslinks on the degradation characteristics of macroporous hydrogels based on P(PF-co-EG) over a 12-week period. The porosity was varied by the concentration of SB and AH, the precursors to the carbon dioxide porogen. The molecular weight between crosslinks was varied by the ratio of P(PF-co-EG) to PEG-DA. Degradation was assessed via gravimetry, mechanical testing, and μCT.
8.2 Materials and Methods

8.2.1 Poly(propylene fumarate-co-ethylene glycol) Synthesis

The block copolymer poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) was synthesized in a two step reaction as described previously [Behravesh et al., 2002b]. Poly(propylene fumarate) (PPF) was first synthesized from diethyl fumarate (Acros, Pittsburgh, PA) and propylene glycol (Fisher, Pittsburgh, PA). The block copolymer was synthesized from the transesterification reaction of the PPF and methoxy poly(ethylene glycol) (mPEG) of number average molecular weight of 1960 (Aldrich, Milwaukee, WI). The number average molecular weight of the resulting P(PF-co-EG) copolymer was found to be 4760 as measured by NMR (Bruker, Zurich, Switzerland).

8.2.2 Determination of Molecular Weight Between Crosslinks

Thin films were synthesized from P(PF-co-EG) and poly(ethylene glycol)-diacrylate (PEG-DA) of nominal molecular weight of 700 (Aldrich). The polymers were dissolved in double distilled water on a 33% by volume basis. The redox initiation system for the crosslinking reaction consisted of an oxidizer, ammonium persulfate (APS) (Acros), combined with a reducer, ascorbic acid (AH) (Aldrich). APS and AH were added sequentially to the prepolymer each at a final concentration of 0.01 M.

Hydrogels were crosslinked between two glass plates separated by a 1 mm spacer overnight at 37°C and then swollen in phosphate buffered saline (PBS) (Sigma) for 24 hours. Swollen hydrogel films were either cut into dog-bone shapes using a stainless
steel cutter for tensile testing, or into thin disks 10 mm in diameter for equilibrium polymer volume fraction calculations.

Glass slides were glued using cyanoacrylate to the ends of the swollen hydrogel dog-bones to provide a stable gripping surface. The samples prepared for tensile testing were 33 mm in length, 6 mm wide, and between 1 and 2 mm in thickness at the narrowest point. The exact dimensions were measured prior to testing using a digital caliper. An Instron 5500 series tabletop load frame (Instron Corporation, Canton, MA) was utilized at a crosshead speed of 10 mm/min to test each sample in tension until failure. The stress and strain data were recorded.

The equilibrium polymer volume fraction of the swollen hydrogel ($v_{2,s}$) was calculated as follows:

$$v_{2,s} = \frac{V_p}{V_{g,s}}$$

The volume of the equilibrium swollen hydrogel, $V_{g,s}$, was measured after weighing samples, equilibrium swollen in double distilled water (DDW), in air ($W_{a,s}$) and in cyclohexane (Fisher) ($W_{n,s}$) using a hanging pan balance as follows:

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

where $\rho_n$ is the density of the non-solvent cyclohexane ($\rho_n = 0.774 \text{ g/cm}^3$). Samples were air dried overnight, vacuum dried for 24 hrs, and then weighed ($W_{a,d}$). The volume of the dried hydrogel, $V_p$, was calculated as follows:
\[ V_p = \frac{W_{s.d}}{\rho_p} \]

where \( \rho_p \) is the density of the polymer and estimated to be 1.15 g/cm\(^3\) [Shung et al., In Press].

The Young’s modulus (\( E \)) was determined from the tensile testing of dog-bone shaped hydrogels and calculated as the slope of the initial linear portion of the stress/strain curve. The molecular weight between crosslinks (\( M_c \)) was estimated from the following equation assuming an affine deformation [Sperling, 1992]:

\[ M_c = \frac{3\rho_p RT}{E} \nu_{2.3}^{1/3} \]

where \( R \) is the gas constant and \( T \) is the temperature. The molecular weight between crosslinks of the macroporous hydrogels was estimated from these non-porous thin film samples.

### 8.2.3 Fabrication of Macroporous Hydrogels

Macroporous hydrogels were composed of the block copolymer P(PF-co-EG) and PEG-DA in the same ratios used for thin films. The polymers were dissolved in DDW on a 33\% by volume basis. Sodium bicarbonate (SB) (Aldrich), APS, and AH were added sequentially. The reaction of SB and acid produced the porogen, carbon dioxide, while APS and AH were utilized as redox initiators. Each component was well mixed with the polymer solution by utilizing a vortexer before the subsequent addition of the next
component. A typical formulation consisted of 8 g of total polymer in the appropriate volume of aqueous solution including SB, APS, and AH, and crosslinked in a 2.5 cm diameter by 12 cm height vial. Samples were left overnight at 37°C prior to equilibrium swelling.

8.2.4 Experimental Design

A full factorial experimental design was employed to assess the effects of porosity via SB-AH concentrations [Behravesh et al., 2002a] and crosslinking density via P(PF-co-EG):PEG-DA ratio [Shung et al., In Press]. High and low values for the two parameters are listed in Table 8-1a. The formulation compositions are numerated in Table 8-1b. APS concentration was held constant at 0.25 M. The SB-AH precursors to the carbon dioxide porogen, were varied congruently to result in high and low levels of porosity.

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<tr>
<th>Table 8-1a. High and low levels for the parameters tested in a two-level full factorial design</th>
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<td>Level</td>
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<td>High (+)</td>
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<td>Low (-)</td>
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<th>Table 8-1b. Combination of experimental variables in a two-level full factorial design</th>
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<td>Group</td>
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The degradation of P(PF-co-EG) based macroporous hydrogels was examined in vitro in PBS at a constant pH of 7.4. The buffer was changed every 8 hours for the first 2 days, daily for the rest of the week, and weekly for the remaining 12 weeks. Samples were kept at 37°C during the course of the degradation with gentle agitation (60 rpm). Gravimetry, mechanical testing, and microcomputed tomography were performed after initial swelling at day 1 (week 0), and at 2, 4, 8, and 12 weeks.

8.2.5 Gravimetry

Macroporous hydrogel samples were cut with a razor blade to approximately 4 mm in height and with a cork borer to 9 mm in diameter. Each sample was air dried overnight and vacuum dried for 24 hrs to remove all residual water. Samples were weighed individually and swollen in excess PBS. At the end of each time point, the samples were removed from PBS, the surface water was removed with the aid of weight paper, and their weight was measured. Each sample was then vacuum dried and reweighed. The normalized weight loss was calculated as:

\[
\text{normalized weight loss} = \left( \frac{W_{S,d} - W_{S,rd}}{W_{S,d}} \right) \cdot 100\%
\]

where \( W_{S,d} \) is the initial weight of the dried sample before degradation and \( W_{S,rd} \) is the weight of the redried sample at the end of that time point. The total water content was measured as follows:
normalized total water content = \( \frac{W_{S,a} - W_{S,rd}}{W_{S,a}} \times 100\% \)

where \( W_{S,a} \) is the weight of the equilibrium swollen sample at that time point.

### 8.2.6 Mechanical Testing

Macroporous hydrogels were cut with a razor blade horizontally to approximately 1.5 cm in height and then vertically into four parts. Each segment was placed in a 50 ml centrifuge tube with approximately 30 ml PBS. A confined compression apparatus was constructed to test the compressive modulus of the hydrogels. A thin metal annulus (6 mm inner diameter, 12 mm height) with a sharp edge was used as a confined sample holder. At each time point, samples were removed from PBS and cut using the sample holder to fill the chamber without any compression or distortion of the sample. The sample holder was submerged in a beaker of PBS so that testing was carried out in a wet environment. The compressive testing was performed with a Materials Testing System (MTS Corporation, Eden Prairie, MN) equipped with a plunger (5.8 mm diameter) moving at 1 mm/min to compress the hydrogel within the sample holder to 0.5 mm/mm strain. The load and displacement were recorded throughout the test to obtain the stress/strain curve. The compressive modulus was evaluated as the slope of the initial linear portion of the curve. Mechanical testing was carried out 5 times \((n=5)\) for each sample group at each time point.
8.2.7 Microcomputed Tomography

The morphology of macroporous hydrogels was analyzed via microcomputed tomography (Scanco Medical, μCT 80, Bassersdorf, Switzerland) by measuring their porosity, surface area density, pore wall thickness, and pore edge length (Figure 8-1). Three cylindrical specimens (9 mm in diameter, 4 mm in height) were divided vertically with a razor blade into 6 segments. Each segment was labeled and individually placed in a 50 ml centrifuge tube in excess PBS. At the end of each time point, one of the segments from each of the three cylinders was removed, placed in DDW for 2 hrs, frozen in liquid nitrogen, and freeze-dried for 72 hrs to preserve the structure. By this method, three samples were evaluated (n=3) for each group at each time point.

![Microcomputed tomography scan](image)

**Figure 8-1.** A microcomputed tomography scan of a macroporous hydrogel consisting of 5 sections represented by different gray intensities (750 μm in length by 750 μm in height by 50 μm in depth) illustrating the morphological parameters (A) pore wall thickness and (B) pore edge length.
Specimens were scanned at an energy of 50 kV and an intensity of 80 μA to adequately reconstruct the macroporous hydrogels. A long integration time (300 msec) was utilized to enhance the signal to noise ratio because of the low x-ray adsorption coefficient of polymeric materials. The specimens were scanned at a resolution of 10 μm voxels [Ulrich et al., 1999]. Thresholding and height analyses were conducted to optimize the test conditions and sample size for quantification of morphological parameters. The threshold level was varied between 25 and 60, and the change in porosity, surface area, and surface area to hydrogel volume ratio was measured. A minimum change in these parameters with varying threshold level suggested an optimized threshold level suitable for quantification. The height was varied to quantify the effect of change in computed volume on the morphological parameters. The height of the computed cylinder was varied from 1 mm to 2 mm corresponding to 100 to 200 slices. For analysis of samples, the height of the cylinder was kept constant.

8.2.8 Hydrogel Equilibrium Water Content

The equilibrium water content of hydrogels was calculated from gravimetry and μCT measurements. The total water content, which was measured on a weight basis (\(twc\)), was converted to a volume basis (\(twc_v\)) by accounting for the density of the polymer fraction as follows:

\[
twc_v = \frac{twc}{twc + (1 - twc) \rho_p}
\]
The hydrogel equilibrium water content, corresponding to the volume of water entrapped within the hydrogel at equilibrium, was defined on a per volume basis as follows:

\[
\text{Hydrogel equilibrium water content} = \frac{twc \_ v - \text{porosity}}{1 - \text{porosity}}
\]

where the porosity was determined by µCT. The standard deviation of the hydrogel equilibrium water content was calculated by propagation of error of total water content and porosity data.

8.2.9 Statistical Analysis

Data were analyzed with analysis of variance (ANOVA) using Tukey's HSD (highly statistically different) multiple comparison test. An alpha level of 0.05 was defined as significant. Reported measurements are presented as means ± standard deviations (n=4 for gravimetry, n=5 for mechanical testing, and n=3 for microcomputed tomography).

8.3 Results

8.3.1 Hydrogel Synthesis

The compositions were selected to result in macroporous hydrogels with varying porosities and crosslinking densities. The M_c of the macroporous hydrogels was estimated from non-porous hydrogels. The equilibrium polymer volume fraction of swollen hydrogel films synthesized with a P(PF-co-EG):PEG-DA ratio of 1:1 and 3:1
was $0.40 \pm 0.00$ and $0.36 \pm 0.01$, respectively. In addition, the Young’s modulus was measured to be $6.3 \pm 0.6$ and $3.3 \pm 0.6$ MPa for hydrogels synthesized with a P(PF-co-EG):PEG-DA ratio of 1:1 and 3:1, respectively. The $M_c$ was estimated to be $1000 \pm 100$ and $1880 \pm 320$ g/mol for hydrogels synthesized with a P(PF-co-EG):PEG-DA ratio of 1:1 and 3:1, respectively.

### 8.3.2 Gravimetry

The normalized weight loss and total water content of the macroporous hydrogels are shown in Figure 8-2. There was a significant weight loss compared to the week 0 (Day 1) samples after 8 weeks for Group 1, 8 weeks for Group 2, 4 weeks for Group 3, and 12 weeks for Group 4 (p<0.05). Day 1 samples were used to measure the sol fraction of the hydrogel. Weight loss due to the sol fraction was measured for all four formulations after the first day in PBS. The weight loss due to degradation alone after 12 weeks, with propagation of error, for Groups 1 and 2 was $22.3 \pm 8.4$ and $20.5 \pm 9.1 \%$, respectively, while for Groups 3 and 4 was $12.4 \pm 1.4$ and $12.9 \pm 6.0 \%$, respectively. Weight loss for Groups 1 and 2 were significantly greater than for Groups 3 and 4 (p<0.05).
Figure 8-2. Weight loss (a) and total water content (b) of macroporous hydrogels of Group 1 (■), Group 2 (□), Group 3 (●), and Group 4 (○) defined in Table 8-1 over 12-weeks of degradation. Error bars represent means ± standard deviations for n=4.
8.3.3 Mechanical Testing

Representative stress/strain curves, obtained under confined compression, over the time course of the experiment are shown in Figure 8-3. The initial linear portion of the stress/strain graph was utilized to determine the compressive modulus (Figure 8-4). The formulation with low porosity and low $M_C$ (Group 4) had the greatest initial compressive modulus $610 \pm 150$ kPa. Groups 1 and 3 showed significant loss in modulus after 4 weeks of degradation compared to the initial time point ($p<0.05$). The modulus was below detectible levels and could not be calculated for Group 1 after 8 weeks of degradation.

![Stress/strain curves](image)

**Figure 8-3.** Representative stress/strain curves of macroporous hydrogels of Group 4 under confined compression after (a) 0, (b) 4, and (c) 12 weeks of *in vitro* degradation. Dashed lines represent the initial linear portion of the stress/strain curve.
Figure 8-4. Confined compressive modulus of macroporous hydrogels of Group 1 (■), Group 2 (□), Group 3 (●), and Group 4 (○) defined in Table 8-1 over 12-weeks of degradation. Inset is a magnification of Groups 1-3. Error bars represent means ± standard deviations for n=5.

8.3.4 Microcomputed Tomography

The threshold level and sample volume were optimized to quantitatively analyze the morphology of the specimens (Figure 8-5). The threshold level at which the minimum change in the examined properties occurred was utilized for analysis of the samples (threshold level = 42). Varying the height of the computed samples resulted in no trends or local minima. A cylindrical volume 1.5 mm in diameter and 1 mm in height (100 slices) was selected to quantitate the morphological parameters of the macroporous hydrogels.
Figure 8-5. Determination of change in porosity ($\blacksquare$), surface area ($\bullet$), and surface area to hydrogel volume ($\square$) with varying threshold level (a) and height of sampled cylinder (b).
A representative μCT reconstruction (16° angled and top view) of Groups 3 and 4 described in Table 8-1b at the initial time point (week 0) and after 12 weeks of degradation is presented in Figure 8-6. Quantitatively, prior to degradation, there was no significant difference in porosities synthesized with the same SB-AH concentrations; however, the porosities of Group 1 (0.88 ± 0.02) and Group 3 (0.88 ± 0.04) were significantly higher from Group 2 (0.81 ± 0.03) and Group 4 (0.78 ± 0.02) (p<0.05). Similarly, the surface area density of Group 1 (70 ± 16 cm⁻¹) and Group 3 (70 ± 17 cm⁻¹) were significantly lower from the surface area density of Group 2 (118 ± 10 cm⁻¹) and Group 4 (126 ± 13 cm⁻¹) (p<0.05). There was no statistical difference between the pore wall thickness for any of the groups examined at the initial time point (p>0.05). However, the pore edge length values for the initial time point followed the same trends as the porosity data. The pore edge length of Group 1 (285 ± 14 μm) and Group 3 (289 ± 22 μm) were significantly larger from the pore edge length of Group 2 (156 ± 7 μm) and Group 4 (173 ± 18 μm) (p<0.05). Over the time course of the degradation study for each sample group, there was no significant change in porosity, surface area density, and pore edge length (p>0.05) (Figure 8-7). There was a general trend of increased pore wall thickness with degradation.
Figure 8-6. Representative μCT scans of Group 3 (a) and Group 4 (b) macroporous hydrogels (see Table 8-1) after 0 and 12 weeks of *in vitro* degradation. Sampled areas were 2.5 mm in diameter and 1.5 mm in thickness with a spatial resolution of 10 μm.
Figure 8-7. Variation in porosity (a), surface area density (b), pore wall thickness (c), and pore edge length (d) evaluated at 0 (□), 2 (■), 4 (□□), 8 (□□), and 12 (□□) weeks of degradation for macroporous hydrogels described in Table 8-1. Error bars represent means ± standard deviations for n=3.
Figure 8-7. (Continued)
8.3.5 Hydrogel Equilibrium Water Content

The total water content, measured by gravimetry, and the porosity, determined by μCT, were used to calculate the water content of the swollen hydrogel for each macroporous specimen (Figure 8-8). There was a general trend for Groups 2, 3 and 4 in that there was an increase in the water content of the hydrogels with the degradation time; however, the propagation of error in the measurements did not allow for any statistical comparisons.

Figure 8-8. Hydrogel equilibrium water content evaluated at 0 ( ), 2 ( ), 4 ( ), 8 ( ), and 12 ( ) weeks of degradation for macroporous hydrogels described in Table 8-1. Error bars represent means ± standard deviations calculated by propagation of error of total water content and porosity data.
8.4 Discussion

The objective of this study was to evaluate the effect of porosity and molecular weight between crosslinks on the in vitro degradation characteristics of P(PF-co-EG) based macroporous hydrogels. Degradation was evaluated through gravimetry, mechanical testing, and µCT. The initial formulations selected showed no significant difference in porosities or surface area densities between Groups 1 and 3 or Groups 2 and 4. These groups were designed to have the same porosity. In addition, the molecular weight between crosslinks were significantly different with varying P(PF-co-EG):PEG-DA ratio. The M_c of the macroporous hydrogels was estimated from thin non-porous hydrogel films. Previous studies have shown that the main factor affecting the M_c of P(PF-co-EG) based hydrogels is the ratio of P(PF-co-EG) to PEG-DA [Shung et al., In Press]. Therefore, the effect of porosity and molecular weight between crosslinks of these P(PF-co-EG) based macroporous hydrogels on the examined properties could be analyzed utilizing a 2^2 full factorial approach [Neter et al., 1996].

Scaffolds and thin films fabricated from linear polyesters, such as poly(lactic-co-glycolic acid) (PLGA), have been previously shown to degrade primarily by a bulk degradation mechanism via hydrolysis of the ester bond, exhibiting an initial decrease in the mechanical properties without a simultaneous decrease in the mass or the overall dimensions of the sample [Lu et al., 2000b]. P(PF-co-EG) and poly(lactic acid-co-ethylene glycol) (P(LA-co-EG)) based hydrogel thin films, which also rely on the degradability of their ester bond, have also been shown to degrade in this bulk fashion [Martens et al., 2001; Metters et al., 1999; Suggs et al., 1998b]. However, the
macroporous hydrogel degradation characteristics described in this study were found to be different from macroporous scaffolds of hydrophobic polymers including those fabricated from PLGA. The most fundamental difference was mass loss from the onset of degradation not typically found in hydrophobic polymers that undergo bulk degradation.

There are two important factors that can contribute to the mass loss due to degradation of these hydrogels. The hydrogels were designed to have a significant portion of dangling PEG chain ends, which do not contribute to the overall network structure. Terminal PEG chains of the P(PF-co-EG) copolymer are linked to the network via a hydrolytically degradable ester bond. With degradation, these dangling chain ends can be released from the network. In addition, P(PF-co-EG) is a highly unsaturated block copolymer, with the PPF block containing one unsaturated fumarate bond per repeating unit. Potentially, not all of the fumarate functional groups are utilized in the crosslinking reaction. Fumaric acid and propylene glycol, the degradation products of the uncrosslinked PPF repeat unit, can be released from the network, contributing to the weight loss.

All groups had a decrease in compressive modulus throughout the degradation period. Together, weight loss and decreased mechanical properties suggest degradation of the network chains. With a looser network structure, an increase in the water content of the hydrogel was observed. For this study, the equilibrium water content was calculated by using both gravimetry and μCT data. The equilibrium water content of the hydrogel, excluding the water content of the macropores, was found to increase with degradation time. In addition, there was a general trend of increasing pore wall thickness, also
suggesting an increase in the hydrogel water content. The increase in water uptake and pore wall thickness is further evidence of bulk degradation, where, with degradation, the less crosslinked network structure leads to greater water uptake by the hydrogel.

The morphology of the macroporous hydrogel was characterized by μCT examination. The use of μCT enabled the accurate description of architectural features of macroporous hydrogels by calculating the porosity, surface area density, pore wall thickness, and pore edge length. The computed volume for analysis was the same for samples throughout the degradation time. The porosity did not significantly change for any of the groups. Isotropic expansion due to degradation, as evidenced by the increase in water content, would not result in a change in the porosity. In contrast, increase in porosity has been shown to be indicative of surface erodible systems [Batycky et al., 1997; Hanes et al., 1998]. In addition, these macroporous hydrogels provided a large surface area, $70 \pm 16$ and $70 \pm 17$ cm$^{-1}$ scaffold for Groups 1 and 3 and $118 \pm 10$ and $126 \pm 13$ cm$^{-1}$ scaffold for Groups 2 and 4, respectively. Following the trends for porosity, there was no change in the surface area density with degradation.

The most significant factor affecting macroporous hydrogel degradation was the molecular weight between crosslinks. Samples with a relatively large $M_C$ had the greatest mass loss after 12 weeks with a significant decrease in compressive moduli. The hydrogel degradability was dependent on its crosslinking density. With greater crosslinking density, more ester bonds have to be hydrolyzed to produce soluble chains, ultimately resulting in slower degradation.

The porosity did not affect the degradation of P(PF-co-EG) based macroporous hydrogels which exhibited a weight loss profile similar to that of thin film hydrogels.
[Metters et al., 1999]. The difference between the low and high porosity samples (Groups 1 and 3 vs. Groups 2 and 4) was in the pore edge length, an indicator of the average pore size or spacing of the hydrogel [Gibson et al., 1988]. The initial pore edge lengths were $285 \pm 14$ and $290 \pm 25 \ \mu m$ for Groups 1 and 3, and $157 \pm 7$ and $173 \pm 18 \ \mu m$ for Groups 2 and 4, respectively. There was no significant change of the pore edge length with degradation for all groups.

One limitation of this study was that the differences in porosity were due to difference in pore size; the macroporous hydrogels studied had similar initial pore wall thicknesses, between $41 \pm 2$ to $45 \pm 2 \ \mu m$. Potentially, the pore wall thickness could be an important parameter for the degradation of these macroporous hydrogels [Lu et al., 2000b]. Another limitation of this study was the estimation of the molecular weight between crosslinks from thin film samples. Because AH participates in both the free-radical and pore formation reactions [Behravesh et al., 2002a], the initiator concentrations utilized in the synthesis of thin films were different from those used for the synthesis of the macroporous hydrogels. Although previous work showed that the P(PF-co-EG):PEG-DA ratio has the most significant effect on the crosslinking density of these hydrogels, the initiator concentration does have an effect on the crosslinking density [Shung et al., In Press]. The $M_C$ values measured from non-porous samples were only an estimate for the macroporous samples.

The experimental results of increased weight loss, increased water content, decreased compressive modulus, and increased pore wall thickness with degradation time all support a bulk degradation mechanism for the P(PF-co-EG) macroporous hydrogels. Through this degradation mechanism, the surface of the material remains intact while the
bulk of the material undergoes hydrolytic degradation. This is in contrast to surface erodible systems where degradation occurs by continual renewal of the surface making cell attachment to the material difficult [Temenoff et al., 2001]. This bulk degradation mechanism renders these hydrogels with the reported surface area densities and pore edge lengths suitable for consideration for tissue engineering applications.

8.5 Conclusions

The degradation of macroporous hydrogels based on P(PF-co-EG) was dependent on the molecular weight between crosslinks and independent of the porosity. There was significant mass loss, increased total water content, and decreased mechanical properties for all formulations during the 12-week study. Porosity and surface area density of the hydrogels did not significantly change over the time course of the degradation. In addition, there was a trend of an increase in the hydrogel equilibrium water content and pore wall thickness with degradation. The results indicate that macroporous hydrogels based on P(PF-co-EG) degrade via a bulk mechanism.
CHAPTER 9*

Three-Dimensional Culture of Marrow Stromal Osteoblasts in Biomimetic Poly(propylene fumarate-co-ethylene glycol)-Based Macroporous Hydrogels

Chapter Abstract

This study assessed the ability of biomimetic poly(propylene fumarate-co-ethylene glycol)-based hydrogels to sustain the differentiation of marrow stromal cells (MSCs) to the osteoblastic phenotype in vitro. Macroporous hydrogels based on poly(propylene fumarate-co-ethylene glycol) with and without covalently linked RGD cell adhesive peptide were synthesized and seeded with rat MSCs suspended in media or in a type I collagen solution. Cells suspended in media were found to adhere to RGD-modified, and not to unmodified hydrogels. Cells suspended in a collagen solution were entrapped after collagen gelation and proliferated independent of the peptide modification of the hydrogel. Hydrogel modification with RGD peptide was sufficient to allow for the adhesion and differentiation of MSCs to the osteoblastic phenotype in the presence of osteogenic culture supplements. MSCs seeded with a collagen gel onto RGD-modified macroporous hydrogels showed a significant increase in cell numbers after 28 days of culture from 15,200 ± 2,000 to 208,600 ± 69,700 cells. Moreover, significant calcium deposition was apparent after 28 days of culture in RGD-modified hydrogels for cells

* This chapter is from: Behravesh and Mikos (Submitted): J. Biomed. Mater. Res.
suspended in a collagen gel in comparison to cells suspended in media, \(3.47 \pm 0.26\) compared to \(0.82 \pm 0.20\) mg Ca\(^{2+}\) per scaffold. Confocal microscopy revealed that MSCs suspended in a collagen gel and cultured on RGD-modified hydrogels for 28 days were adhered to the surface of the hydrogel while MSCs suspended in a collagen gel and cultured on unmodified hydrogels were located within the pores of and not in direct contact with the hydrogel surface. The results demonstrate that these biomimetic hydrogels facilitated the adhesion and supported the differentiation of MSCs to the osteoblastic phenotype in the presence of osteogenic culture media.

9.1 Introduction

Macroporous constructs for bone tissue engineering have served as scaffolds for tissue ingrowth and made use of hydrophobic biomaterials including metals, ceramics, and synthetic polymers [Devin et al., 1996; Hollinger et al., 1986; Ishaug-Riley et al., 1997; Vehof et al., 2001]. These materials depend on protein adsorption on their surface to mediate cellular interaction [El-Ghannam et al., 1999; Kilpadi et al., 2001; Tzampazis et al., 2000]. Recently, biomimetic materials have been used to modulate cell function and guide tissue regeneration. One method of synthesizing biomimetic materials is by modifying hydrogels, which are at first non cell adhesive, with cell adhesive segments of extracellular matrix molecules, the most widely used of which is the ubiquitous RGD amino acid sequence [Ruosslahti et al., 1987].
Hydrogels based on poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) have been shown to be biocompatible exhibiting minimal fibrous encapsulation in vivo [Suggs et al., 1999b]. In addition, these hydrogels have been formed to macroporous scaffolds with up to 90% porosity allowing guided tissue formation [Behravesh et al., In Press-a]. By themselves, these hydrogels prevent cell adhesion [Shung et al., In Press]; incorporating integrin-binding peptides covalently linked to the bulk of the hydrogels has been shown to promote cell adhesion via specific receptor-ligand interactions [Behravesh et al., In Press-b].

In previous studies, we have investigated the adhesion and migration of primary marrow stromal cells (MSCs) on bulk-modified fumarate-based hydrogels with cell adhesive peptide sequences [Behravesh et al., In Press-b; Shin et al., 2002]. MSCs can be differentiated to osteoblast-like cells with appropriate soluble signaling molecules including glucocorticoids [Bruder et al., 1994; Maniatopoulos et al., 1988; Ozawa et al., 1996]. The differentiation of these osteoprogenitor cells to the osteoblastic phenotype has been well characterized with several markers including alkaline phosphatase (ALPase) activity, an early differentiation marker, and mineralization, a late differentiation marker [Aubin, 1996; Stein et al., 1996].

This study asked the following question: can hydrogels support long term MSC proliferation and osteoblast differentiation? To answer this question, MSCs were cultured on macroporous P(PF-co-EG)-based hydrogels with a covalently linked RGD cell adhesive peptide. MSCs were seeded to the hydrogels using culture media or a type I collagen gel as a carrier and their proliferation and differentiation characteristics were assessed with several assays.
9.2 Materials and Methods

9.2.1 Polymer Synthesis

The block copolymer poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) was synthesized as previously described [Behravesh et al., 2002b]. Briefly, bis (2-hydroxy propyl) fumarate was synthesized by the reaction of diethyl fumarate (Acros, Pittsburgh, PA) and propylene glycol (Fisher, Pittsburgh, PA). The product was then transesterified to form poly(propylene fumarate) (PPF). A number average molecular weight of 1700 (PI=1.8) was measured by gel permeation chromatography with a calibration curve based on polystyrene standards.

P(PF-co-EG) block copolymers were then synthesized by the transesterification reaction of the above synthesized PPF and methoxy poly(ethylene glycol) (mPEG) of nominal molecular weight of 2000 (Aldrich, Milwaukee, WI) (number average molecular weight of 1990 as measured by nuclear magnetic resonance spectroscopy (NMR)). The copolymer was then dissolved in methylene chloride (Fisher), purified by precipitation in ethyl ether (Fisher), isolated by filtration, and dried under vacuum. The number average molecular weight of the resulting copolymer was 4870 with 1.8 terminal PEG blocks as measured by NMR.

Acryloyl-poly(ethylene glycol)-GRGDS (Ac-PEG-RGDS) was synthesized from acryloyl poly(ethylene glycol) N-hydroxysuccinimide (Ac-PEG-NHS) (Shearwater, Huntsville, AL) of nominal molecular weight of 3400 and the oligopeptide sequence GRGDS (Bachem, Torrance CA), respectively. Ac-PEG-NHS and the oligopeptide were dissolved separately in 5 mM sodium bicarbonate buffer, pH 8.2, at a final concentration
of 75 mg/ml and 1 mg/ml, respectively. A molar ratio of 2 Ac-PEG-NHS to peptide was used to ensure a complete reaction of the RGDS peptide. Sodium bicarbonate was removed via dialysis using a molecular weight cutoff dialysis membrane of 2000 (Spectrum Laboratories, Rancho Dominguez, CA) with 5 double distilled deionized water (DDW) changes over a course of 2 days. The polymer-peptides were lyophilized to remove water for 48 hours and stored below -4°C until use.

9.2.2 Scaffold Fabrication

The P(PF-co-EG) macroporous hydrogels were fabricated using a simultaneous gas foaming, free-radical crosslinking approach [Behravesh et al., 2002a]. P(PF-co-EG) was mixed with poly(ethylene glycol)-diacrylate (PEG-DA) of nominal molecular weight of 700 (Aldrich) on a 1:1 wt% basis and dissolved with vigorous mixing in DDW at a final polymer concentration of 66 wt%. For some formulations, Ac-PEG-RGDS was mixed and dissolved in the polymer solution, the concentration of which was determined to result in a peptide concentration of 1000 nmol/cm³. Sodium bicarbonate (Aldrich) (80 mg per ml of initial polymer solution) was added to the polymer solution. Subsequently, ammonium persulfate (0.25 M) (Acros) and ascorbic acid (0.1 M) (Aldrich) were added to the solution and mixed to form the macroporous hydrogels. The sample groups examined are described in Table 9-1.
Table 9-1. Description of experimental groups examined.

<table>
<thead>
<tr>
<th>Group</th>
<th>RGDS Bulk Concentration (nmol/cm³)</th>
<th>Cell Carrier</th>
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<tr>
<td>1</td>
<td>1000</td>
<td>Culture Media</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>Culture Media</td>
</tr>
<tr>
<td>3</td>
<td>1000</td>
<td>Collagen Gel</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>Collagen Gel</td>
</tr>
</tbody>
</table>

Samples were kept at 37°C overnight, cut into thin disks approximately 2-3 mm in thickness, and hydrated in PBS for 48 hours. The swollen scaffolds were then cut using a cork borer to 7 mm in diameter, transferred to excess 70% ethanol overnight, and placed on a shaker table (60 rpm). Subsequently, samples were washed with PBS with 1% antibiotics/antimycotic (Invitrogen, Carlsbad, CA) at least 3 times over the course of 2 days in preparation for use in cell culture.

9.2.3 Marrow Stromal Osteoblast Isolation and Culture

Marrow stromal cells were isolated as previously described [Behravesh et al., In Press-b; Maniatopoulos et al., 1988]. Briefly, femurs and tibias of 125-150 g male Wistar rats (Harlan, Indianapolis, IN) were excised aseptically. Bone marrow was flushed out using cell culture media (defined below) expelled form an 18 1/2 gauge needle. Cell culture suspensions were prepared by repeated aspiration of the cells through an 18 1/2 gauge followed by a 22 1/2 gauge needle. The cell suspension from two animals was plated onto one triple flask (Fisher) having a surface of 500 cm² and cultured using Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen), 10% fetal bovine serum.
(FBS) (Gemini Bio-product, Calabasas, CA), 1% antibiotic/antimycotic (Invitrogen) and the osteogenic supplements $10^{-8}$ M dexamethasone (Sigma), $10^{-3}$ M β-glycerol phosphate (Sigma), and 50 µg/ml L-ascorbic acid (Sigma).

All cell culture was conducted with this culture media containing the osteogenic supplements, referred to as complete media, at 37°C in a humidified incubator with an atmosphere of 5% CO$_2$ and 95% humidity. Media was exchanged three days after initial cell plating onto the triple flask. After 6 days of cell culture, the cells were washed twice with PBS, enzymatically lifted with exposure to 20 ml of 0.05% Trypsin/0.53 mM EDTA (Sigma) for 5 min, neutralized with 10 ml of FBS, diluted with DMEM, centrifuged, and resuspended for use in the study. Experiments conducted for this study utilized cells at this first passage.

9.2.4 Cell Culture and Analysis of Macroporous Hydrogel Scaffolds

Macroporous hydrogels were removed from PBS and placed in 6-well ultra-low attachment polystyrene plates (Fisher) for long-term cell culture. Control samples received no cells. For the experimental groups, $6.25 \cdot 10^4$ MSCs were added at their first passage in one of two methods: 1) cells suspended in media loaded on top of the scaffold and 2) cells suspended in a collagen type I solution at a concentration of 1 mg/ml prepared with 0.8 ml of medium, 2 ml of neutralizing medium (DMEM, 10% FBS containing 0.1 N sodium hydroxide), 4 ml of rat tail type I collagen (3.40 mg/ml; BD Biosciences, Bedford, MA), and 6.8 ml of MSC suspension ($1.25 \cdot 10^4$ cell/ml) in media. Cells suspended in a collagen solution were kept on ice and used immediately by top loading.
A 100 μl aliquot of the above cell suspensions, containing media or collagen, was added to four formulations of macroporous hydrogels as described in Table 9-1. Seeded constructs were kept in the incubator for 3 hrs followed by the addition of 5 ml of media to each well. The media were changed every 3 days for the first week and every 2 days until the completion of the study. Constructs were collected after 1, 4, 7, 14, 21, and 28 days in culture following initial cell seeding.

At each time point, samples were thoroughly washed with PBS three times, removed from the six well plates, and placed in 5 ml snap cap tubes. One ml of DDW was added to the sample and frozen below −20°C until analyzed. Samples underwent two freeze thaw cycles with sonication on ice for 30 min after each cycle prior to analysis.

9.2.4.1 DNA analysis

Double stranded DNA content of the scaffolds was analyzed using a PicoGreen assay (Molecular probes) according to the manufacturer’s instructions. Fifty μl of sample, 100 μl of buffer and 150 μl of PicoGreen solution were mixed in a 96 well plate. Analysis of double stranded DNA was performed using an automated plate reader (FLx 800, Bio-Tek Instruments, Winooski, VT) with an emission/excitation wavelength of 485/528 nm. Measured fluorescence intensities were correlated to cell numbers using a calibration curve constructed with MSCs of known concentration in the range of 0 to 5.5·10⁵ cells per ml and adjusted by the blank controls.
9.2.4.2 Alkaline phosphatase analysis

Samples of the solution were assayed for alkaline phosphatase (ALPase) activity using p-nitrophenyl phosphatase (Sigma) according to the manufacturer’s instructions. In a 96-well plate 80 µl of sample, 20 µl of working buffer, and 100 µl of substrate solution were added to each well. The 96-well-plates were incubated for 1 hour at 37°C in a cell culture incubator, and subsequently, 100 µl of 0.3 N sodium hydroxide were added to each well to stop the reaction. Standards were prepared in concentrations ranging from 0 – 25 µM p-nitrophenol. Absorbance was measured for each well at 405 nm on a UV-VIS plate reader (Powerwave x, Bio-Tek Instruments, Winooski, VT). The concentration of p-nitrophenol was measured, correlated to the ALPase activity per hour of incubation time, and normalized to the cell number.

9.2.4.3 Calcium content

The total calcium present was determined using the Sigma Diagnostic Kit #587 as a measure of the total mineralized deposits in each scaffold. Samples were prepared according to the manufacturer’s instructions. A wide tip pipet was utilized to accurately aliquot a representative 250 µl of each sample into a 5 ml snap cap tube. Acetic acid (250 µl, 1N) was added to each sample and left for 24 hrs to dissolve the mineralized deposits. From each sample, 10 µl were placed into wells of a 96-well plate. Aliquots of 300 µl of the assay working solution, prepared by mixing equal parts of the calcium binding reagent and calcium buffer reagent provided in the kit, were added to each sample well. The 96-well plate was incubated for 10 min at room temperature and then
the absorbance was measured at 575 nm on a plate reader (Powerwave x). Measured absorbance intensities were correlated to the amount of equivalent Ca\textsuperscript{2+} using a calibration curve made with CaCl\textsubscript{2} solutions of known concentrations in the range of 0 to 300 µg/ml. Samples were diluted as necessary to fall into the calibration range.

9.2.5 Confocal Microscopy

Macroporous hydrogels were prepared for confocal microscopy after 28 days of culture. The samples were fixed in 10% formalin solution for at least 30 min and then washed with PBS to remove the fixative. PicoGreen dye (0.1%) was added to each sample and incubated for 15 minutes prior to imaging. A confocal microscope (Zeiss LSM Axiovert, Carl Zeiss, Germany) was utilized to take an image in the z-plane, perpendicular to the plane of the microscope image. The region of interest was selected from this z-plane image to include either the surface or internal pores beginning with a top section at least 100 µm below the surface of the macroporous hydrogels. Depth projection micrographs were obtained from 16 horizontal sections imaged at a depth distance of 20 µm from each other. Images were pseudo-colored to show depth as a function of color.

9.2.6 Statistical Analysis

Unseeded macroporous hydrogels cultured similarly to the sample groups served as negative controls. All data were analyzed with analysis of variance (ANOVA) using Tukey’s HSD (highly statistically different) multiple comparison test where an alpha
level of 0.05 was defined as significant. Reported measurements are represented as means ± standard deviation where n=3 for all measurements.

9.3 Results

9.3.1 Macroporous Hydrogel Scaffold Preparation

Addition of ascorbic acid resulted in the dissolution of sodium bicarbonate to carbon dioxide with concurrent free-radical production ultimately resulting in macroporous hydrogels. These macroporous scaffolds were fabricated with and without bulk-modified RGDS peptide with no clear difference in morphology between the formulations. The swollen hydrated scaffolds utilized for cell culture were 3-4 mm in thickness and 7 mm in diameter.

9.3.2 Marrow Stromal Cell Seeding

During the initial 3 hour incubation time at 37°C, after cell seeding, the scaffolds were left undisturbed in the incubator to allow the cells to attach for the formulations containing cells suspended in media, and allow for the gelation of the collagen entrapping the cells within the scaffold pores for cells suspended in collagen. For Group 1 samples, RGD-modified macroporous hydrogels seeded with cells suspended in media, light microscopy inspection revealed that the MSCs were attached and spread on the surface of the RGD-modified hydrogels, while for Group 2 samples, unmodified hydrogels seeded in the same manner, the cells were rounded. In contrast, for Groups 3 and 4, where the
cells were entrapped within a collagen gel, light microscopy revealed that the cells were suspended throughout the collagen gel filling the pores of the macroporous hydrogel with no clear difference between the two groups.

9.3.3 Cell Proliferation

The total number of cells cultured on the macroporous hydrogels was quantified using a DNA assay over the time course of the study (Figure 9-1). For hydrogels seeded with cells suspended in collagen (Groups 3 and 4) there was an increase from 15,200 ± 2,000 and 15,000 ± 2,400 cells after initial seeding to 208,600 ± 69,700 and 218,700 ± 52,800 cells after 28 days of culture, respectively. Peptide-modified hydrogels seeded with cells suspended in media (Group 1) did not show significant proliferation from 17,900 ± 6,700 cells present after initial cell seeding. Macroporous hydrogels without peptide modification seeded with cells suspended in media (Group 2) showed minimal adhered cells after 4 days of culture (2,000 ± 700 cells) and below detectable levels, similar to unseeded hydrogel controls, after 7 days of culture.

9.3.4 Alkaline Phosphate Activity

The ALPase activity, an indicator of the osteoprogenitor cell’s commitment to the osteoblastic phenotype, was normalized by the total cell number as quantified by the DNA assay for each sample individually (Figure 9-2). Group 2 samples did not contain measurable amount of ALPase activity, similar to blank controls, and were not included in the analysis. For Group 1, there was a significant increase in ALPase activity after 4 days in culture (24 ± 1 pmol/hr/cell) compared to day 1 (11 ± 4 pmol/hr/cell) followed
by a decrease in activity, returning to day 1 levels after 28 days in culture (17 \( \pm \) 6 pmol/hr/cell). Groups 3 and 4 showed similar increase and subsequent decrease in ALPase activity, with a significant increase after 7 (71 \( \pm \) 7 pmol/hr/cell) and 4 days in culture (86 \( \pm \) 10 pmol/hr/cell), respectively, and returned activity to day 1 levels (38 \( \pm \) 14 and 42 \( \pm \) 26 pmol/hr/cell) after 14 days (28 \( \pm \) 7 and 29 \( \pm \) 8 pmol/hr/cell) in culture for both groups, respectively.

![Diagram](image)

**Figure 9-1.** Cellularity of macroporous hydrogel scaffolds for 1 (II), 4 (Z), 7 (□), 14 (○), 21 (□), and 28 (□) days of *in vitro* culture. Groups examined are described in Table 9-1. Data are shown as means \( \pm \) standard deviation, and evaluated from three samples. The symbol (*) represents a significant difference between time points of the same group.
Figure 9-2. Alkaline phosphatase activity per cell for 1 (III), 4(□), 7(□), 14(□), 21(III), and 28 (□) days of *in vitro* culture. Groups examined are described in Table 9-1. Group 2 was excluded from analysis due to a lack of detectable alkaline phosphatase activity. Data are shown as means ± standard deviation, and evaluated from three samples. The symbol (*) represents a significant increase in alkaline phosphatase activity from day 1 and (**) represents a significant decrease in alkaline phosphatase activity from the * time point.
9.3.5 Mineralization

The total calcium content of each sample was measured on a per scaffold (Figure 9-3a) or per cell (Figure 9-3b) basis. Significant calcium deposition was found after 14 days of culture in complete media for Groups 3 and 4 (0.68 ± 0.24 and 0.40 ± 0.19 mg Ca$^{2+}$ per scaffold, respectively). After 28 days of culture for Groups 1, 3, and 4, there were 0.82 ± 0.20, 3.47 ± 0.26, and 2.95 ± 0.63 mg Ca$^{2+}$ per scaffold, and none detected at any time point for Group 2. On a per cell basis, calcium deposition for Groups 1 (2.9 ± 0.7 · 10$^{-5}$ mg Ca$^{2+}$ per cell) was larger than for Groups 3 and 4 (1.2 ± 0.2 and 1.4 ± 0.3 · 10$^{-5}$ mg Ca$^{2+}$ per cell, respectively).

9.3.6 Confocal Microscopy

Cell distribution after 28 days of cell culture was visualized by staining the DNA of the cells using PicoGreen dye. Representative confocal depth projection images containing the top surface (Figure 9-4 a-c) and the internal pores (Figure 9-4 d-f) of the macroporous hydrogels were obtained for Groups 1, 3, and 4 and showed an uneven distribution of cells at the top surface of the scaffolds. Visual inspection revealed that Group 1 samples had a lower cell density when compared to Group 3 and 4 samples. The cells seeded on scaffolds modified with the RGD peptides (Groups 1 and 3) were in direct contact with the hydrogel. In contrast, the cells cultured on unmodified scaffolds (Group 4) had no intimate contact with the hydrogel. In addition, the distribution of the cells in the internal pores of the scaffold were qualitatively different, where Groups 1 and 3 had cells directly on the internal pore surface of the hydrogels while Group 4 contained cells within the collagen gel filled pores.
Figure 9-3. Calcium content of scaffold due to mineralization normalized per scaffold (a) and per cell (b) for 7(□), 14(□), 21(□□), and 28 (□□) days of in vitro culture. Groups examined are described in Table 9-1. Group 2 was excluded from analysis due to a lack of detectable calcium. Data are shown as means ± standard deviation, and evaluated from three samples. The symbol (*) represents a significant difference between time points of the same group. The symbol (**) represents a significant difference between groups of the same time point.
Figure 9-4. Representative 28-day confocal depth projection micrographs of the surface (a-c) and internal pores below the surface (d-f) of macroporous hydrogels. Macroporous hydrogels shown are modified with the RGDS peptide in the bulk seeded with cells suspended in media (a and d), modified with the RGDS peptide in the bulk and seeded with cells suspended in a collagen gel (b and e), and unmodified and seeded with cells suspended in a collagen gel (c and f). Color bar represents the depth of the cells from the initial point of scan. The orange arrow points to the hydrogel while the white arrow points to the cell layer.
9.4 Discussion

The objective of this study was to assess the potential of biomimetic P(PF-co-EG)-based hydrogels for supporting osteoblastic differentiation as biomaterials for bone tissue engineering. Previous studies have illustrated the effect of initiator and porogen concentrations on synthesized hydrogel scaffold morphology and formation of interconnected pore structure [Behravesh et al., 2002a; Behravesh et al., In Press-a]. Additional studies have showed the effect of covalently linked RGDS concentration on adhesion and migration of marrow derived osteoblasts cultured in complete media on thin film P(PF-co-EG)-based hydrogels [Behravesh et al., In Press-b]. In this study, the long-term differentiation of the MSCs cultured in complete media on three-dimensional macroporous hydrogels was evaluated.

The mechanism of cell adhesion to RGD peptides has long been known to be integrin dependent [Carvalho et al., 1998; Geissler et al., 2000; Kantlehner et al., 2000; Moursi et al., 1996; Rezania et al., 1999b; Ruoslahti et al., 1987]. In addition, these cell surface integrin receptors and their interactions with collagen [Mizuno et al., 1997; Moursi et al., 1996; Moursi et al., 1997] and fibronectin [Carvalho et al., 1998; Moursi et al., 1996; Moursi et al., 1997] containing the RGD sequence have been previously found to be essential to osteoblastic differentiation. In this study, the use of hydrogel scaffolds with covalently linked RGD peptide was found to not only allow for the adhesion of osteoprogenitor cells, but also allow for their differentiation to the osteoblastic phenotype when cultured in complete media containing osteogenic supplements. MSCs seeded
directly using media (Group 1) and seeded using a collagen carrier (Groups 3 and 4) on the macroporous hydrogels exhibited typical phenotypic markers of osteoblastic cells.

However, the cell seeding method of the scaffolds had a significant impact on the proliferation of the cells. Group 1 hydrogels modified with the cell adhesive RGD peptide did allow for cell adhesion; however, the total cell numbers did not significantly increase for the duration of the study. In comparison, the total cell numbers seeded using a collagen carrier increased significantly after 28 days of culture suggesting proliferation of the initially seeded cells. Potentially, matrix production was a necessary step for the proliferation of the cells on Group 1 hydrogels that contained only the minimum necessary peptide sequence (RGD) for cell adhesion. It is further believed that Group 1 hydrogels did not facilitate initially the adsorption of matrix produced by adhered cells mediating cell proliferation and differentiation, which is corroborated by previous results of negligible cell adhesion on films of similar unmodified hydrogels or modified hydrogels with an RDGS scrambled peptide [Shung et al., In Press; Behravesh et al., In Press-b]. Typically, proliferation of osteoprogenitor cells has been shown to occur prior to matrix maturation and final differentiation [Stein et al., 1996]. The need of matrix production prior the proliferation could have prevented the initial proliferative phase of the osteoprogenitor cells. Once matrix was produced, the cells were committed to late differentiation [Stein et al., 1996], limiting cell proliferation. This is in contrast to Group 3 and 4 hydrogels containing cells entrapped within a collagen gel where the local collagen environment of the osteoprogenitor cells allowed for their proliferation prior to differentiation.
The cells cultured on Groups 1, 3, and 4 showed typical up-regulation and down-regulation of ALPase activity as well as deposition of calcium, both suggesting the successful differentiation to the osteoblastic phenotype. The difference between Group 1 and Groups 3 and 4 was the extent and time scale of differentiation. Alkaline phosphatase levels for all of the groups examined was up-regulated at the same time point, after 4 days of culture; however, the magnitude of ALPase activity was approximately 2 times higher for cells seeded in the collagen gel than in media. In addition, down-regulation of ALPase activity, signaling the initiation of the mineralization phase [Stein et al., 1996], occurred much later (day 28) for Group 1 samples in comparison to Group 3 and 4 samples (day 14). Moreover, significant calcium deposition, which is a measure of mineral production and a marker for the late differentiation of osteoblastic cells, was found at a later time point on Group 1 samples (day 28) compared to Group 3 and 4 samples (day 14). This time lag for Group 1 could be due to less matrix present to induce cell differentiation and mineralization also resulting in decreased proliferation rates. In addition, the positive role of collagen type I for the differentiation of osteoblasts has been well documented and could account for the greater magnitude of ALPase activity and faster calcium deposition for Groups 3 and 4. These results suggest that cell adhesion on biomimetic hydrogels is necessary but not sufficient condition for subsequent cell function and that kinetics of cell differentiation depends on the extent of available environmental signals.

One limitation of this study was the modification of the hydrogel with only one concentration of RGD peptide. Previous studies on P(PF-co-EG) based hydrogel thin films showed that an RGD peptide bulk concentration of 1000 nmol/cm$^3$ was sufficient to
promote the adhesion of 91 ± 10% of the initially seeded marrow derived cells after 12 hrs incubation time [Behravesh et al., In Press-b]. Although a lower concentration was also shown to promote cell adhesion, a concentration of 1000 nmol/cm³ was selected to allow the cells to adhere strongly to the hydrogel. Future studies will investigate the effect of RGD concentration of bulk-modified macroporous hydrogels on the differentiation of osteoprogenitor cells.

The localization of the cells was visualized using confocal microscopy. For groups with adhered cells, there were a disproportionately larger number of cells at the top surface of the hydrogel compared to its interior after 28 days of culture (Figure 9-4a-c). This observation could be due to diffusional limitations of nutrients [Ishaug et al., 1997; Ishaug-Riley et al., 1997; Marra et al., 1999] after mineralization of the top surface of the hydrogel. Differences in the localization of cells were also apparent when examining the internal pores of hydrogels for Groups 3 and 4. In both groups, initially, the collagen gel allowed for the entrapment of the cells within the pores of the macroporous hydrogel. The macroporous hydrogel served as a scaffold preventing the collapse of the collagen gel. However, after 28 days of culture, cells cultured on Group 4 samples were not in direct contact and within the pores of the hydrogel. This is consistent with hydrogels not modified with cell adhesive peptide seeded using media (Group 2) which did not allow for cell adhesion as previously found for cells cultured on thin films [Behravesh et al., In Press-b]. MSCs cultured on Group 4 were able to proliferate solely due to their entrapment within the collagen gel. In contrast, MSCs cultured on Group 3 samples were preferentially adhered to the pore surfaces of the hydrogel. This localization of the cells to the internal surface of the RGD-modified hydrogels and not within the collagen-filled
pores could be advantageous for tissue engineering strategies that depend on the ingrowth of surrounding tissue into the pores of the scaffold.

9.5 Conclusion

MSCs cultured on biomimetic P(PF-co-EG)-based macroporous hydrogels with covalently linked RGD peptide differentiated to phenotypically osteoblastic cells when cultured in media containing osteogenic supplements. The extent of osteoblastic differentiation, as measured by calcium deposition, was dependent on the cell carrier and was greater for MSCs seeded with a collagen type I gel than in culture media. Moreover, MSCs seeded with a collagen gel showed increased proliferation and enhanced up-regulation of ALPase activity compared to those seeded in media. In addition, peptide-modified hydrogels promoted the localization of MSCs on their internal pore surfaces, whereas unmodified hydrogels only supported MSC culture within their pores with a collagen gel carrier.
Bibliography


