RICE UNIVERSITY

Development of Thermally-Crosslinked Hydrogels as Injectable Cell Carriers for Orthopaedic Tissue Engineering

by

Johnna Sue Temenoff

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

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

[Signatures]

Antonios G. Mikos, J.W. Cox Professor, Chair, Bioengineering

Kyriacos A. Athanasiou, Professor Bioengineering

Michael C. Gustin, Associate Professor Biochemistry and Cell Biology

Richard G. LeBaron, Associate Professor Biology, UT – San Antonio

Michael J. Miller, Professor Plastic Surgery, MD Anderson Cancer Center

HOUSTON, TEXAS

AUGUST, 2003
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3122555
Copyright 2004 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346
ABSTRACT

Development of Thermally-Crosslinked Hydrogels as Injectable Cell Carriers for Orthopaedic Tissue Engineering

by

Johnna S. Temenoff

Synthetic hydrogel materials based on oligo(poly(ethylene glycol) fumarate) (OPF) were developed and characterized as injectable cell carriers for orthopaedic tissue engineering. Through alteration of the poly(ethylene glycol) molecular weight used in the synthesis of the OPF macromer, swelling and mechanical properties of the resulting crosslinked hydrogels could be controlled. These hydrogels were characterized in both the swollen and dry states, leading to calculation of their mesh sizes, which varied depending on the OPF type used in crosslinking. In addition, it was found that these gels could be laminated during crosslinking, with each layer having distinct mechanical properties.

Before their use as injectable cell carriers, the cytotoxicity of all OPF hydrogel precursor molecules, including radical initiators and their derivatives, was evaluated using rat marrow stromal cells as a model cell type. Results indicated that the overall pH of the formulation, as well as length of exposure to the components, had significant effects on cell viability. Using this information, an initiator was identified which remained near neutral pH in cell culture media and resulted in crosslinking of two types of OPF hydrogels in 8 min at 37°C.

The optimized OPF formulations were then used to investigate effects of changes in hydrogel swelling properties and media supplements on osteogenic differentiation of
encapsulated rat marrow stromal cells. After 28 days of \textit{in vitro} culture, evidence of cellular differentiation was found in all sample types, indicating that the encapsulation procedure did not have a detrimental effect on the ability of the marrow stromal cells to form bone-like tissue. In the presence of osteogenic supplements, OPF hydrogels with greater swelling promoted embedded MSC differentiation over those that swelled less. In all specimens examined, areas of mineralized matrix were obvious many microns away from the cells, indicating that the hydrogel mesh size was large enough to allow diffusion of matrix components throughout the material. These results demonstrate the great potential of OPF hydrogels as injectable carriers for delivery of cells to a variety of complex orthopaedic defects.
ACKNOWLEDGEMENTS

To my mother, who has taught me to ask "Why?"
and my father, who has taught me to ask "How?"

To document here all that I have learned during the course of my Ph.D. work, or all those who have helped me along the way, would be impossible. As I look back on the ups and downs of the past five years, I am extremely appreciative of the continual guidance and inspiration of my thesis advisor, Dr. Antonios G. Mikos, who has always reminded me of the true reason for our research. I would also like to express my gratitude to my thesis committee, whose expertise has been invaluable in my studies. To my labmates and friends, who have withstood more than their fair share of my complaints (and my domestic disasters!), I thank you. Without your counsel and assistance, neither my thesis, nor my life in Houston, would have been possible.

Finally, words cannot express my deep gratitude for my parents’ support over these many years. I thank them both for enduring a quarter century of incessant questions, and look forward with them to a future filled with exploration.
# Table of Contents

Abstract ii  
Acknowledgements iv  
Table of Contents v  
List of Tables vii  
List of Figures viii  
List of Abbreviations xi  

Chapter I: Background: Injectable Biodegradable Materials for Orthopaedic Tissue Engineering 1  
A. Abstract 1  
B. Introduction 1  
C. Bone Tissue Engineering 4  
D. Cartilage Tissue Engineering 13  
E. Conclusions 16  
F. Figures 17  

Chapter II: Objectives 22  

Chapter III: Effect of Poly(Ethylene Glycol) Molecular Weight on Tensile and Swelling Properties of Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels 24  
A. Abstract 24  
B. Introduction 25  
C. Materials and Methods 27  
D. Results 34  
E. Discussion 36  
F. Conclusions 42  
G. Tables and Figures 44  

Chapter IV: Effect of Drying History on Swelling Properties and Cell Attachment to Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels 53  
A. Abstract 53  
B. Introduction 54  
C. Materials and Methods 57  
D. Results 61  
E. Discussion 66  
F. Conclusions 69  
G. Tables and Figures 71
Chapter V:  *In Vitro* Cytotoxicity of Redox Radical Initiators for Crosslinking of Oligo(Poly(Ethylene Glycol) Fumarate) Macromers
A. Abstract 77
B. Introduction 78
C. Materials and Methods 80
D. Results 84
E. Discussion 91
F. Conclusions 98
G. Tables and Figures 100

Chapter VI: Effect of Swelling Properties of Oligo(Poly(Ethylene Glycol) Fumarate) Hydrogels and Addition of Media Supplements on *In-Vitro* Osteogenic Differentiation of Encapsulated Marrow Stromal Cells
A. Abstract 107
B. Introduction 107
C. Materials and Methods 109
D. Results 116
E. Discussion 121
F. Conclusions 128
G. Tables and Figures 130

Chapter VII: Conclusions 142

Chapter VIII: Bibliography 145
**LIST OF TABLES**

| Table 3.1: | Results of swelling studies for OPF hydrogels after 6 hrs in ddH₂O. | 44 |
| Table 4.1: | Results comparing effects of drying methods on OPF hydrogel swelling properties. | 71 |
| Table 5.1: | Change in pH (from control) of individual initiator components and their combinations over time. | 100 |
| Table 6.1: | Change in pH (from control) of initiator components. | 130 |
| Table 6.2: | Number- (Mₙ) and weight-average (Mₘ) molecular weight of synthesized oligomers as determined by gel permeation chromatography. | 131 |
| Table 6.3: | Times to onset and completion of gelation for OPF 10K and 3K with the radical initiators APS/TEMED (25 mM). | 132 |
| Table 6.4: | Fold swelling and theoretical sol fraction for OPF 10K and 3K hydrogels crosslinked with APS/TEMED. | 133 |
LIST OF FIGURES

Figure 1.1: Diagram of the photopolymerizable dimethacrylated polyanhydrides currently under investigation for bone repair. 17

Figure 1.2: Poly(propylene fumarate) (PPF), an injectable polymer that can crosslink in situ with appropriate mechanical properties to fill defects in cancellous bone. 18

Figure 1.3: Chemical structure of poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG), used as an injectable carrier for chondrocytes to repair cartilage. 19

Figure 1.4: Dimethacrylated poly(ethylene oxide) (PEODM), a polymer that can be transdermally photopolymerized with chondrocytes to form new cartilage. 20

Figure 1.5: Diagram of the structure of poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), a hydrogel currently studied for cardiovascular applications, but that could be modified for use in cartilage repair. 21

Figure 3.1: Schematic of crosslinking reaction that occurs with OPF and PEG-DA using the water soluble initiator system APS/AA. 45

Figure 3.2: Steps in formation of laminated hydrogels for tensile testing. 46

Figure 3.3: Tensile modulus of OPF hydrogels of various PEG molecular weights with and without the presence of an interface. 47

Figure 3.4: Strength at fracture of OPF hydrogels of various PEG molecular weights with and without the presence of an interface. 48

Figure 3.5: Toughness of OPF hydrogels of various PEG molecular weights with and without the presence of an interface. 49

Figure 3.6: Percent elongation at fracture of OPF hydrogels with and without the presence of an interface. 50

Figure 3.7: Swelling ratio of OPF hydrogels at 6 hrs. 51

Figure 3.8: Image of biphasic composite hydrogels before and after swelling in ddH$_2$O overnight. 52
Figure 4.1: Methods for swelling studies.

Figure 4.2: Fold swelling and sol fraction of OPF hydrogels with various drying histories over 3-4 cycles of repeated drying and swelling.

Figure 4.3: Representative DSC graphs of hydrated OPF 10K and OPF 1K hydrogels that had been dried before swelling and the corresponding linear OPF molecules.

Figure 4.4: Percent of seeded human dermal fibroblasts attached to tissue culture polystyrene and OPF hydrogels after 4 and 24 hours.

Figure 4.5: Fluorescent microscope images of OPF surfaces after 24 hours of cell attachment.

Figure 5.1: Chemical structure of the species involved in OPF crosslinking.

Figure 5.2: pH change from control and corresponding marrow stromal cell viability for initiator components and their combinations (10-500 mM).

Figure 5.3: Fluorescent microscopy images of marrow stromal cells after 2 hrs continuous exposure to initiator components and treated with LIVE/DEAD reagent.

Figure 5.4: Time to onset and completion of gelation as assessed by rheometry.

Figure 6.1: Fold swelling and sol fraction of OPF hydrogels without cells over 28 days at 37°C in cell culture media.

Figure 6.2: Cell number per sample for OPF-MSC constructs after various days in culture.

Figure 6.3: Alkaline phosphatase activity per sample for OPF-MSC constructs after various days in culture.

Figure 6.4: Cumulative osteopontin secretion per sample for OPF-MSC constructs over 28 days in culture.

Figure 6.5: Calcium content per sample for OPF-MSC constructs after various days in culture.
Figure 6.6: Calcium content per sample for 10K OPF-MSC constructs after 21 and 28 days in culture, depicted with reference to particular cell isolation.

Figure 6.7: OPF-MSC constructs after 7 and 21 days of \textit{in-vitro} culture.

Figure 6.8: OPF-MSC constructs after 28 days of \textit{in-vitro} culture.
LIST OF ABBREVIATIONS

$\alpha$  Elongation

$\phi$  Hydrogel sol fraction

$\eta^*$  Complex viscosity

$\kappa$  Weight fraction of polymer in solution just before crosslinking

$\rho_o$  Density of hexane (0.66 g/cm$^3$)

$\tau$  Tensile stress

$\nu_{h,s}$  Polymer volume fraction in the swollen hydrogel

$\nu_{h,r}$  Polymer volume fraction in hydrogel after crosslinking but before swelling

$\xi$  Hydrogel mesh size

10K-  OPF 10K hydrogel cultured without dexamethasone

10K+  OPF 10K hydrogel cultured with dexamethasone

3K-  OPF 3K hydrogel cultured without dexamethasone

3K+  OPF 3K hydrogel cultured with dexamethasone

4EDMAB  Ethyl-4-$N,N$-dimethyl aminobenzoate

AA  Ascorbic acid

AD-ES  Hydrogel samples dried in air, vacuum-dried, and then swollen

ALP  Alkaline phosphatase

ANOVA  Analysis of variance

APS  Ammonium persulfate

Asc  Sodium ascorbate

Asc-2  Magnesium ascorbate-2-phosphate

ASTM  American Society for Testing and Materials

BCP  Biphasic calcium phosphate

$C_0$  Original number of cells added to the cell culture well

$C_{2r}$  Mass concentration of polymer in solution before crosslinking

CC  Calcium carbonate (CaCO$_3$)

CDHA  Calcium deficient hydroxyapatite (Ca$_4$(HPO$_4$)(PO$_4$)$_2$OH)

CMP  Calcium magnesium phosphate (Ca$_4$Mg$_5$(PO$_4$)$_6$)

$C_n$  Characteristic ratio
CPH  1,6-bis(p-carboxyphenoxy) hexane  
CPP  1,3-bis(p-carboxyphenoxy) propane  
CQ  Camphorquinone  
CSH  Calcium sulfate hemihydrate (CaSO₄·½H₂O)  
C_1  Number of cells on hydrogel surface after culture  
DCDP  Dicalcium phosphate dihydrate (CaHPO₄·2H₂O)  
DCPA  Dicalcium phosphate anhydrous (CaHPO₄)  
ddH₂O  Distilled, deionized water  
dex  Dexamethasone  
DMEM  Dulbecco's Modified Eagle Medium (cell culture medium)  
DSC  Differential scanning calorimetry  
ECM  Extracellular matrix  
e-PTFE  Expanded poly(tetrafluoroethylene)  
ES  Hydrogel samples swollen to equilibrium  
ES-AD  Hydrogel samples swollen and then dried in air  
ES-VD  Hydrogel samples swollen and then vacuum-dried  
EthD-1  Ethidium homodimer-1 (dye for nonviable cells)  
FDA  U.S. Food and Drug Administration  
F_{dc}  Avg(flourescence_{dead control}) - Avg(background fluorescence_{dye, no cells})  
F_{ds}  Fluorescence_{sample} - Avg(background fluorescence_{dye, no cells})  
F_{lc}  Avg(flourescence_{live control}) - Avg(background fluorescence_{dye, no cells})  
F_{ls}  Fluorescence_{sample} - Avg(background fluorescence_{dye, no cells})  
FuCl  Fumaryl chloride  
GAG  Glycosaminoglycan  
GPC  Gel permeation chromatography  
GRGD  Gly-Arg-Gly-Asp peptide sequence  
GTR  Guided tissue regeneration  
HA  Hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂)  
HDFs  Human dermal fibroblasts  
I+1, I+2  Isolations of rat bone marrow stromal cells cultured with dexamethasone
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1, I-2</td>
<td>Isolations of rat bone marrow stromal cells cultured without dexamethasone</td>
</tr>
<tr>
<td>IPNs</td>
<td>Interpenetrating networks</td>
</tr>
<tr>
<td>L</td>
<td>Length of the narrow portion of dog-bone specimen before fracture</td>
</tr>
<tr>
<td>l</td>
<td>Weighted average of the bond lengths of C-C and C-O bonds</td>
</tr>
<tr>
<td>L₀</td>
<td>Original length of the narrow portion of dog-bone specimen</td>
</tr>
<tr>
<td>M_c</td>
<td>Molecular weight between crosslinks</td>
</tr>
<tr>
<td>MCPM</td>
<td>Monocalcium phosphate monohydrate (Ca(H₂PO₄)₂·H₂O)</td>
</tr>
<tr>
<td>MMA</td>
<td>Methylmethacrylate</td>
</tr>
<tr>
<td>Mₙ</td>
<td>Number-average molecular weight</td>
</tr>
<tr>
<td>M_r</td>
<td>Molecular weight of the oligomer repeating unit</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>Marrow stromal cell</td>
</tr>
<tr>
<td>N</td>
<td>Number of links between crosslinks</td>
</tr>
<tr>
<td>NaPS</td>
<td>Sodium persulfate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>OCP</td>
<td>Octacalcium phosphate (Ca₄(H₂PO₄)₂(PO₄)₃·5H₂O)</td>
</tr>
<tr>
<td>OPF 10K</td>
<td>Oligo(poly(ethylene glycol) fumarate) with nominal PEG molecular weight ~10,000</td>
</tr>
<tr>
<td>OPF 4K</td>
<td>Oligo(poly(ethylene glycol) fumarate) with nominal PEG molecular weight ~4,600</td>
</tr>
<tr>
<td>OPF 3K</td>
<td>Oligo(poly(ethylene glycol) fumarate) with nominal PEG molecular weight ~3,000</td>
</tr>
<tr>
<td>OPF 1K</td>
<td>Oligo(poly(ethylene glycol) fumarate) with nominal PEG molecular weight ~1,000</td>
</tr>
<tr>
<td>OPF</td>
<td>Oligo(poly(ethylene glycol) fumarate)</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>P(PF-co-EG)</td>
<td>Poly(propylene fumarate-co-ethylene glycol)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCPH</td>
<td>Poly(1,6-bis(p-carboxyphenoxy)hexane)</td>
</tr>
<tr>
<td>PCPP</td>
<td>Poly(1,3-bis(p-carboxyphenoxy)propane)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEG-DA</td>
<td>Poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PEODM</td>
<td>Poly(ethylene oxide) dimethacrylate</td>
</tr>
<tr>
<td>PI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PPF</td>
<td>Poly(propylene fumarate)</td>
</tr>
<tr>
<td>PSA</td>
<td>Poly(sebacic acid)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl alcohol)</td>
</tr>
<tr>
<td>Q</td>
<td>Hydrogel volume swelling ratio</td>
</tr>
<tr>
<td>$\bar{r}_e^2$</td>
<td>End-to-end distance of unperturbed polymer chains</td>
</tr>
<tr>
<td>R</td>
<td>Gas constant (8.31 kPa L/mol K)</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp peptide sequence</td>
</tr>
<tr>
<td>S1</td>
<td>Samples dried after fabrication and then swollen</td>
</tr>
<tr>
<td>S2'</td>
<td>Samples swollen after fabrication, dried and then swollen again</td>
</tr>
<tr>
<td>SA</td>
<td>Sebamic acid</td>
</tr>
<tr>
<td>T</td>
<td>Temperature at which tensile testing occurred</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate (Ca$_3$(PO$_4$)$_2$)</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue culture polystyrene</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethyilsilane</td>
</tr>
<tr>
<td>TTCP</td>
<td>Tetracalcium phosphate (Ca$_4$(PO$_4$)$_2$O)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VD-ES</td>
<td>Hydrogel samples vacuum-dried and then swollen</td>
</tr>
<tr>
<td>$W_{s,a}$</td>
<td>Sample weight in air after swelling in ddH$_2$O and vacuum drying</td>
</tr>
<tr>
<td>$W_{s,x}$</td>
<td>Sample weight in air after crosslinking</td>
</tr>
<tr>
<td>$W_{s,s}$</td>
<td>Sample weight in air after swelling in ddH$_2$O</td>
</tr>
<tr>
<td>$W_d$</td>
<td>Weight of hydrogel after swelling and drying</td>
</tr>
<tr>
<td>$W_{d,0}$</td>
<td>Sample dry weight before swelling</td>
</tr>
<tr>
<td>$W_{d,s}$</td>
<td>Sample dry weight after swelling</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$W_i$</td>
<td>Weight of hydrogel after crosslinking</td>
</tr>
<tr>
<td>$W_{n,d}$</td>
<td>Sample weight in hexane after swelling in ddH$_2$O and vacuum drying</td>
</tr>
<tr>
<td>$W_{n,r}$</td>
<td>Sample weight in hexane after crosslinking</td>
</tr>
<tr>
<td>$W_{n,s}$</td>
<td>Sample weight in hexane after swelling in ddH$_2$O</td>
</tr>
<tr>
<td>$W_s$</td>
<td>Weight of hydrogel after swelling</td>
</tr>
</tbody>
</table>
CHAPTER I

BACKGROUND: INJECTABLE BIODEGRADABLE MATERIALS FOR ORTHOPAEDIC TISSUE ENGINEERING

ABSTRACT

The large number of orthopaedic procedures performed each year, including many performed arthroscopically, has led to great interest in injectable biodegradable materials for regeneration of bone and cartilage. A variety of materials have been developed for these applications, including ceramics, naturally-derived substances and synthetic polymers. These materials demonstrate overall biocompatibility and appropriate mechanical properties, as well as promote tissue formation, thus providing an important step towards minimally invasive orthopaedic procedures. This review provides a comparison of these materials based on mechanical properties, biocompatibility and regeneration efficacy. Advantages and disadvantages of each material are explained and design criteria for injectable biodegradable systems are provided.

INTRODUCTION

Every year in the United States, there are approximately 900,000 hospitalizations due to fractures [1]. Of these, over 500,000 are fractures of the lower extremities [1], therefore requiring intervention to restore ambulation. In addition, 36 million Americans suffer from some form of arthritis [1]. Although these maladies affect different tissues (bone and articular cartilage), such statistics demonstrate the vast need for

* This chapter has been published as: J.S. Temenoff, A.G. Mikos, “Injectable biodegradable materials for orthopaedic tissue engineering,” Biomaterials, 2000; vol. 21, p. 2405-2412.
new techniques to treat a wide variety of orthopaedic injuries.

When a lower extremity fracture occurs, immediate mechanical support of the bone is needed for continued limb loading. However, to reduce stress shielding and allow the fracture to heal completely, the support must be removed. Thus, a biodegradable support material would be ideal, as it would eliminate the need for a second surgery to remove the fixation device. Additionally, with the increasing popularity of arthroscopic procedures in orthopaedics, there has been great interest in fixation materials that are injectable as well as biodegradable. Currently, the most commonly-used injectable bone cement is poly(methyl methacrylate) (PMMA), but it suffers from the fact that it is not degraded and that its high curing temperatures can cause necrosis of surrounding tissue [2]. Therefore, further development of alternative injectable materials is necessary, not only for fracture fixation, but also as cell carriers for tissue-engineered regeneration of bone and cartilage. This review will provide an overview of injectable biodegradable biomaterials for use in bone and cartilage regeneration, including design requirements for such materials, as well as comparisons of mechanical properties, biocompatibility and efficacy for each system.

When developing a biomaterial for use in orthopaedics, there are several important requirements that must be satisfied:

**Biocompatibility:** Biodegradable materials for orthopaedic applications, as for any other application, must first be biocompatible. This means that the material must not elicit an unresolved inflammatory response nor demonstrate extreme immunogenicity or cytotoxicity. In addition, because it degrades *in vivo*, this must be true not only for the
intact material and any of its unreacted components, but also for the degradation products [3].

**Mechanical Properties:** Especially important in orthopaedics are the initial mechanical properties of the biomaterial to be implanted. These properties must be as similar as possible to those of the tissue that is to be regenerated. As well as providing proper support in the early stages of healing, graded load transfer is needed later in the process for creation of replacement tissue that is identical to the original [4]. While many mechanical properties should be considered for materials to be used in orthopaedics, including those in compression, tension and torsion, compressive properties are the most relevant for replacement of cancellous bone, while tensile properties are important for cortical bone [5]. Compressive strength will be used to compare mechanical properties of the various materials covered in this review as it is the only parameter reported consistently throughout the literature.

**Promotion of Tissue Formation:** Properties such as amount of void space and degradation time should be chosen to encourage tissue growth and vascularization (if appropriate) within the material. It is important that the degradation rate be coupled to the rate of tissue formation so that the load-bearing capabilities of the tissue are not compromised [3].

**Sterilizability:** As with all implanted materials, orthopaedic materials must be easily sterilizable to prevent infection. The method of sterilization, however, must not interfere with the bioactivity of the materials or alter its chemical composition which could, in turn, affect its biocompatibility or degradation properties [4].
Furthermore, biodegradable materials that are injectable must possess additional properties:

**Setting Time/Temperature Change:** The material should set in several minutes to minimize the length of the procedure while allowing surgeons ample time for placement before hardening [4]. If the setting reaction involves a temperature change, the increase or decrease should be as small as possible to reduce damage to the surrounding tissue.

**Viscosity/Ease of Handling:** Ease of handling is of utmost importance for clinical use of any biomaterial. Therefore, viscous properties must be balanced between the need for the material to remain at the site of injection and the need for the surgeon to easily manipulate its placement.

Recent development of a variety of injectable materials, both ceramic-based and polymeric-based, have fulfilled many of these design criteria for very diverse orthopaedic applications. The results are promising and pave the way for a new era of orthopaedic materials that provide more and better tissue regeneration with less patient discomfort than other techniques currently employed.

**Bone Tissue Engineering**

Both polymeric materials and ceramics have been studied as a means to repair defects in bone. To facilitate comparison between these classes of materials, the focus of this review will be on those systems that have been well-characterized, especially in the area of ceramics where many combinations of calcium-phosphate materials have been tested.
Ceramic Materials

Since 1892, when Plaster of Paris was first used as a bone cement [6], ceramics have often been chosen to aid in fracture fixation and filling of bony defects. They promote bony ingrowth, are biocompatible and harden in situ [7]. Further studies have shown that the most common types of calcium phosphate ceramics, hydroxyapatite (HA, Ca_{10}(PO_4)_{6}(OH)_2) and tricalcium phosphate (TCP, Ca_3(PO_4)_2) have different characteristics in vivo, although both forms have Ca/P ratios within the range known to promote bone ingrowth (1.50-1.67). In general, HA was found to be more osteogenic while TCP was degraded much faster [8, 9].

Using this information, Daculsi et al. developed a biphasic calcium phosphate (BCP), a combination of HA and β-TCP [9]. Particles of BCP (60/40 HA/β-TCP weight ratio) were incorporated at various concentrations in a 2% methyl cellulose carrier gel, so that the material was easily injectable. When implanted in rabbit distal femurs, none of the composites showed unresolved inflammation after ten weeks and bone ingrowth proceeded from the perimeter inward at a greater rate than in BCP blocks alone [10, 11]. This may be because the cellulose spaces the BCP particles apart, providing a ceramic scaffold with voids that favor bone and blood vessel formation [10, 12]. A study using particles of different sizes demonstrated that smaller grains result in faster bone formation, with 50% of the original BCP resorbed after 2 weeks in the small (40-80 μm) grain composite [10]. However, the major drawback to this system is that it has no significant initial mechanical properties. If bone ingrowth were very rapid, this problem may eventually be overcome, but the lack of sufficient initial mechanical characteristics
can also lead to difficulty in maintenance of the composite within the defect during surgery [10].

In response to these concerns, other researchers have chosen to investigate the use of ceramic pastes [7, 13-18]. This type of calcium phosphate ceramics sets in-situ, so that it is initially injectable but after several minutes undergoes non-exothermic setting to form materials with high compressive strength. These calcium phosphates can be divided into four types, depending on what is precipitated during setting: dicalcium phosphate dihydrate (DCDP, CaHPO$_4\cdot$2H$_2$O), calcium magnesium phosphate (CMP, Ca$_4$Mg$_5$(PO$_4$)$_6$), octacalcium phosphate (OCP, Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4\cdot$5H$_2$O), or calcium deficient hydroxyapatite (CDHA, Ca$_9$(HPO$_4$)$_4$(PO$_4$)$_3\cdot$OH) [19]. Of these, the two types that have been studied most extensively are DCPD and CDHA.

A DCPD cement currently under investigation has an initial compressive strength of 25-35 MPa, higher than that of cancellous bone (5-10 MPa [20]) [7, 13]. The cement is formed by mixing β-TCP, monocalcium phosphate monohydrate (MCPM, Ca(H$_2$PO$_4$)$_2\cdot$H$_2$O), and calcium sulfate hemihydrate (CSH, CaSO$_4\cdot$1/2H$_2$O)) in an aqueous solution. The amounts of each component vary depending on the exact properties desired, but is generally about 40 wt.% β-TCP, 13 wt.% MCPM and 10 wt.% CSH [7, 13]. These are available in powder form and can be sterilized with γ-radiation [13].

Cylinders (4.7 mm diameter X 10 mm long) of this cement exhibited overall biocompatibility and were almost completely replaced by bone at 8 weeks after implantation in defects (5 mm diameter) in the distal femoral chondyle of rabbits [13]. When injected, the material was easily shaped and formed a good interface with the existing bone [14]. However, although this ceramic has acceptable mechanical properties
and is quickly replaced by bone, DCPD cements have been found to turn acidic during setting, potentially inducing inflammation around the implantation site [19].

In contrast to the DCPD cements, CDHA cements remain neutral after setting [19]. A material of this type is under development by several investigators [15-18, 21-24]. The cement, a mixture of tetracalcium phosphate (TTCP, Ca₄(PO₄)₂O) and dicalcium phosphate anhydrous (DCPA, CaHPO₄), sets within 5-7 minutes in a sodium hydrogen phosphate solution. The components are sterilizable via γ-radiation and have demonstrated bone formation with no unresolved inflammatory response when implanted in rat tibiae for 8 weeks [16]. Initial compressive strength was 10 MPa for specimens 6 mm in diameter by 3 mm high [15], but premature decay can ensue if the material contacts blood before completely setting [18]. Also, this formulation shows no signs of degradation at 8 weeks [16]. Addition of other materials such as alginate, chitosan and collagen is currently being investigated to resolve these problems [21-24].

A different type of CDHA cement has recently been developed with similar setting characteristics [25-29]. A mixture of MCPPM, TCP and calcium carbonate (CC, CaCO₃) in a sodium phosphate solution, this material has initial compressive strength of 10 MPa. After 12 hours hardening in vivo, the maximum compressive strength of 55 MPa and tensile strength of 2.1 MPa is attained [26]. This value falls between that of cancellous (5-10 MPa) and cortical bone (130-220 MPa) in compression, but, in tension, the cement’s properties are lower than those of native bone (5-10 MPa cancellous, 80-150 MPa cortical [20]).

This material can be sterilized by γ-radiation, shows no extended inflammatory response, and has been observed to cause bone replacement in canine proximal tibial
metaphyseal and distal femoral metaphyseal defects [25]. Because of these properties, the cement has recently been FDA approved for use in fixation of distal radial fractures [30]. Clinical trials using the cement in conjunction with screw fixation for hip fractures improved load transfer and reduced screw cutout [28].

While this material is not currently approved for use in orthopaedics, encouraging clinical trials suggest that it may soon be employed throughout the body. However, it must be noted that in canines, full cement resorption did not occur during the 78 week experimentation period [25]. Concerns about this lengthy resorption time have led to the development of another type of cement that sets endothermically when placed in the body [31]. Calcium phosphate powders, sterilized through γ-radiation, were mixed in saline and implanted in femoral-slot defects in canines. Although initial mechanical properties of the material were not reported, nearly complete resorption was seen 1-2 months after implantation and histology showed lamellar or Haversian bone present in the defects after 12 weeks [31].

Despite such work on cements that are quickly resorbed, many existing calcium phosphate materials degrade very slowly [16, 25], which can lead to decreased bone regeneration at the site of the implant. And, while these cements exhibit good biocompatibility [16, 25, 31] and perform well in compression [7, 13, 15, 26], tensile strengths are still below those found in natural bone [26]. In an effort to address these concerns, researchers have chosen to investigate polymeric materials for use in orthopaedic applications.
**Polymeric Materials**

Polymers can offer some distinct advantages over ceramic materials. Like ceramics, they are injectable and harden *in situ*, but the mechanical properties and degradation times can be more easily tailored with polymers than with calcium phosphate materials [20]. In addition, the widely varied polymer chemistry allows the possibility of functionalization to interact specifically with certain cell types. However, depending on the polymer formulation, they may be less biocompatible and more difficult to sterilize without damage than their ceramic counterparts.

To provide the necessary mechanical strength for use in orthopaedics, injectable polymers must be polymerized or cross-linked *in situ*. This curing is usually initiated either chemically or via the use of light. Recently, a group of photopolymerizable poly(anhydrides) with suitable strength has been developed to fill bony defects [20, 32-34]. These materials are polymers of sebacic acid (SA) alone, or copolymers of SA and 1,3-bis(p-carboxyphenoxy) propane (CPP), or 1,6-bis(p-carboxyphenoxy) hexane (CPH) (see Figure 1.1). The most effective means of photopolymerization for these polymers was found to be 1.0 wt.% camphorquinone (CQ) and 1.0 wt.% ethyl-4-N,N-dimethylaminobenzoate (4EDMAB) with 150 mW/cm² of blue light. This system has been widely used in dentistry and allows penetration of the light to larger depths than UV systems because of the tendency of CQ to quickly photobleach [20, 32].

Depending on the monomer(s) used, the mechanical properties as well as degradation time can be varied. In general, compressive strengths of 30-40 MPa and tensile strengths of 15-27 MPa were obtained, similar to those of cancellous bone [20, 33]. The tensile properties of these polymers are much higher than those of a CDHA
ceramic [26], which may be important to prevent fractures in the time before the implants are replaced by host bone. Poly(sebacic acid) (PSA) degrades quickly (about 54 hours in saline), while poly(1,6-bis(p-carboxyphenoxy)hexane (PCPH) degrades much more slowly (estimated 1 year). Therefore, combinations of different amounts of SA with CPH would result in a polymer with degradation properties custom-designed for a specific application [20, 33]. Because these polymers are surface-eroding, they maintain bulk mechanical properties while undergoing degradation. With either PSA or PCPH, over 70% of the initial tensile modulus remained at 50% mass degradation [33]. To further modulate either degradation time or mechanical properties, these polymers have been photopolymerized with particles or other linear polymers within them, making interpenetrating networks (IPNs). In this case, the additive within the IPN could alter the physical properties of the material to better suit the chosen application [33].

Minimal inflammatory response to the SA/CPP IPN was observed when implanted subcutaneously in rats up to 28 weeks. Loose vascularized tissue had grown into the implant at 28 weeks, with no evidence of fibrous capsule formation [34]. No data have been reported on polymer sterilizability, heat generation during polymerization or polymerization time. However, a 12-week study using a 2.3 mm diameter full-thickness defect in the distal femur of rabbits showed good tolerance of the SA/CPP IPN and osseous tissue formation in the outer zone of some implants [34].

While this family of photopolymerizable polymers has many important characteristics for use in orthopaedics, dependence on light to polymerize the material may be impractical for use in deep crevices occurring in some bones. In these cases, the
investigators suggest the use of a combination of photopolymerized and chemically polymerized materials [32].

In order to eliminate the need for the defect to be exposed to light and work toward a more minimally invasive surgery, other researchers are exploring polymers that, rather than polymerized, are chemically cross-linked in vivo. In this area, a promising candidate is poly(propylene fumarate) (PPF). PPF is an unsaturated linear polyester that can be crosslinked through the fumarate double bond (see Figure 1.2). The degradation products are propylene glycol, poly(acrylic acid-co-fumaric acid), and fumaric acid, a substance which occurs naturally as a part of the Kreb’s cycle [35]. Many methods to synthesize PPF have been explored, and each results in different polymer properties (see [36] for review of these methods) [36]. Cross-linking usually occurs with either methylmethacrylate (MMA) [37, 38] or N-vinyl pyrrolidone monomers [39-41] and benzoyl peroxide as a radical initiator. Depending on the ratio of initiator, monomer, and PPF, the curing time can range from 1-121 minutes [42]. Although the cross-linking reaction is exothermic, it has been shown that temperatures never reach above 48°C during setting as compared to 94°C for a PMMA bone cement [42].

The mechanical properties of PPF can vary greatly according to the synthetic method and the cross-linking agents used. In order to improve these properties for use in orthopaedics, PPF is often combined with particles of ceramic materials such as β-TCP, calcium carbonate, or calcium phosphate [2, 37, 38, 41]. These composite materials exhibit compressive strengths from 2-30 MPa, which is appropriate for replacement of cancellous bone.
PPF degradation time is dependent on polymer structure as well as other components if it is part of a composite material. According to recent in-vitro studies, the time needed to reach 20% original weight ranged from near 84 (PPF/β-TCP composite) to over 200 days (PPF/CaSO₄ composite) [2, 41]. In vivo, β-TCP appears to act as a buffering agent, maintaining local pH and preventing accelerated PPF degradation [43]. Although PPF undergoes bulk degradation, resulting in a decrease in overall mechanical properties with time, its compressive strength actually increases in the short term due to continued cross-linking. Therefore, larger strengths were seen after 3 weeks in saline (9.4 MPa) than when first placed in solution (2.6 MPa) [2].

PPF does not exhibit a deleterious long-term inflammatory response when implanted subcutaneously in rats. Initially, a mild inflammatory response was observed, and a fibrous capsule formed around the implant at 12 weeks [43]. The PPF/β-TCP composite, sterilized by ultra-violet radiation, was implanted in rat tibiae for up to 5 weeks. At this time, the material was observed to be gradually replaced by bone from the perimeter inward [40]. Recent in vitro work provides further information about the osteoconductive properties of PPF/β-TCP by demonstrating that the composite encourages attachment, proliferation and differentiated osteoblastic function of rat marrow stromal cells [44].

To improve the rate and extent of new bone formation, current research includes the addition of osteoblasts to the PPF construct [45]. Another approach involves modification of the PPF with a GRGD (Gly-Arg-Gly-Asp) peptide sequence to encourage host cell attachment and migration once the construct has been injected [46]. New studies with PPF composites include encapsulated growth factors (TGF-β1), which,
rather than improving initial mechanical properties, act to direct cell migration and differentiation within the material [47, 48]. It is PPF's versatility, stemming from its ability to be easily modified, as well as its excellent mechanical properties, that makes this polymer an exciting candidate for future patients needing bone replacement.

**Cartilage Tissue Engineering**

Approaches to cartilage tissue engineering differ significantly from bone tissue engineering, although many of the same considerations for implantable replacements still apply. Like bone, cartilage must withstand compressive loads, but another important function of hyaline cartilage is frictionless movement in the joint [49, 50]. Therefore, the ability to withstand the shear forces at the joint surface is very important. However, mechanical properties of cartilage differ between joints and between different areas of the same joint [51-53] and the minimal necessary mechanical properties for new cartilage have not been determined. For these reasons, biochemical rather than mechanical analysis is often used to assess the extent of cartilage regeneration. In addition, cartilage does not undergo constant remodeling and often demonstrates poor regenerative capacity [54, 55]. Thus, unlike bone, most of the constructs implanted to repair cartilage have included a cellular component.

Because of the differences in needed mechanical properties and the lack of remodeling, ceramics are not appropriate for cartilage applications. In this section, a variety of both naturally-derived and synthetic polymers will be compared for use in cartilage repair, with a focus on biocompatibility and promotion of tissue formation as measures of implant efficacy.
Naturally-derived polymers, such as collagen, fibrin and hyaluronic acid, have often been used as carriers for cells to regenerate various tissues [3, 56-59]. Of these, the two that have been most widely studied for injectable cartilage applications are fibrin glues and alginate gels. Several researchers are exploring the option of injecting fibrinogen and thrombin to form a degradable fibrin mesh that can be used as a scaffold for chondrocytes [58, 59]. Because the patient’s own fibrinogen and thrombin can be used, sterilizability, biocompatibility, and temperature change upon setting are not large concerns. When the cell-fibrinogen-thrombin mixture was injected into defects in horses, hyaline-like cartilage was formed, with more glycosaminoglycan (GAG) and type II collagen present at eight months than in defects that were left untreated [59].

Alginate, a derivative of seaweed, is another option for use in cartilage repair. A liquid, it is injected and crosslinked with calcium to prevent migration from the defect. Alginate can be steam sterilized and the cross-linking reaction does not adversely affect the surrounding tissue [60]. Only mild inflammation was observed in vivo, but these studies were completed in nude mice and the elimination of the animals’ immune system may have reduced the inflammatory response [61]. Although histologic evaluation revealed the architecture of the newly formed tissue to be similar to that of native cartilage, there was little sign of alginate degradation after 12 weeks [60, 61]. An additional concern with the use of alginate is that some forms have been found to be immunogenic [62].

Because of possible complications with antigenicity and obtaining adequate amounts of natural polymers to fill large defects, other investigators have turned to the development of synthetic polymers, such as poly(ethylene oxide) (PEO) (see Figure 1.3).
While sterilization methods were not described, Sims et al. injected a PEO gel with bovine articular chondrocytes into a nude mouse and did not observe any adverse inflammatory response [63]. After 12 weeks, cartilage with histology similar to that of the epiphyseal plate and GAG content approaching that of natural bovine cartilage had been produced [63].

Another synthetic injectable material currently under investigation is a PEO dimethacrylate (PEODM) that can be photopolymerized transdermally (see Figure 1.4). Initial results indicate the constructs can be polymerized in 3 minutes with no harm to imbedded chondrocytes. Specimens explanted from athymic mice at 2, 4, and 7 weeks show cartilage formation with increasing GAG and collagen content [64]. While transdermal photopolymerization may not be possible for many orthopaedic applications, a light source may be provided arthroscopically for some procedures. As in the case of bone, however, the problem of consistent photopolymerization in deep crevices remains.

An alternative may be the use of a material that chemically cross-links in situ. A poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) hydrogel has been developed for use in cardiovascular applications [65, 66] and could be modified for use in a chondrocyte-polymer construct (see Figure 1.5). This material can be sterilized via ultra-violet radiation and has been found to be biocompatible, eliciting an initial inflammatory response in rats that recedes by 21 days, followed by fibrous capsule formation at 12 weeks [67, 68]. Cross-linking resulted in a slight temperature increase (from 37°C to 38.3°C), and short-term studies show no adverse effects of the reaction on endothelial cells injected concurrently with the polymer [69]. These properties indicate that this material is a promising option for future work in cartilage repair.
CONCLUSIONS

Although bone and articular cartilage are very diverse tissues providing different functions within the body, recent work has resulted in new injectable biomaterials with promise to repair both tissues. Like the tissues, the materials are also varied; they can be ceramic, naturally-derived, or based on synthetic polymers. They have different degradation characteristics and can contain imbedded cells or can be used alone. However, all are largely biocompatible and many demonstrate encouraging mechanical properties, an improvement over current injectables. In time, each material may find its own unique application. Taken together, these advances provide a means to lower cost as well as discomfort to persons undergoing a variety of orthopaedic procedures, thus signaling a significant step towards the widely-held ideal of inexpensive, minimally-invasive surgery.
Figure 1.1: Diagram of the photopolymerizable dimethacrylated polyanhydrides currently under investigation for bone repair. PSA: poly(sebamic acid), PCPP: poly(1,3-bis(p-carboxyphenoxy)propane), PCPH: poly(1,6-bis(p-carboxyphenoxy)hexane) [20, 33].
**Figure 1.2:** Poly(propylene fumarate) (PPF), an injectable polymer that can crosslink *in situ* with appropriate mechanical properties to fill defects in cancellous bone [36].
**Figure 1.3:** Chemical structure of poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG), used as an injectable carrier for chondrocytes to repair cartilage [63].
**FIGURE 1.4:** Dimethacrylated poly(ethylene oxide) (PEODM), a polymer that can be transdermally photopolymerized with chondrocytes to form new cartilage [64].
Figure 1.5: Diagram of the structure of poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), a hydrogel currently studied for cardiovascular applications, but that could be modified for use in cartilage repair [65].
CHAPTER II

OBJECTIVES

The overall goal of this work was to develop and characterize synthetic hydrogel materials based on oligo(poly(ethylene glycol) fumarate) (OPF) as injectable cell carriers for orthopaedic tissue engineering. As cells were to be included in the polymer solution as crosslinking occurred, a main concern was the identification of a cytocompatible crosslinking system that allowed specific control of the material properties of the resulting hydrogels. Since the inclusion of progenitor cells, such as those found in the bone marrow, could provide the possibility to regenerate multiple types of orthopaedic tissues, the effect of encapsulation on cellular differentiation was also examined. The studies described here depict characterization of both the OPF hydrogels and the resulting cell-hydrogel constructs. In particular, this work comprised the following specific objectives:

1) To develop a method to create OPF hydrogels with varied swelling and mechanical properties.

2) To characterize the physical properties of the resulting OPF hydrogels, including determination of hydrogel mesh size.

3) To characterize the cytocompatibility of the resulting OPF hydrogels and their constituent components using rat bone marrow stromal cells as a model cell type.

4) To develop a cytocompatible crosslinking formulation for OPF hydrogels.
5) To characterize the effect of changes in swelling properties on osteogenic
differentiation of rat bone marrow stromal cells encapsulated in OPF hydrogels in
vitro.

6) To characterize the effect of media supplements on osteogenic differentiation of
rat bone marrow stromal cells encapsulated in OPF hydrogels in vitro.
CHAPTER III

EFFECT OF POLY(ETHYLENE GLYCOL) MOLECULAR WEIGHT ON TENSILE AND SWELLING PROPERTIES OF OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) HYDROGELS

ABSTRACT

This study was designed to determine the effect of changes in poly(ethylene glycol) (PEG) molecular weight on swelling and mechanical properties of hydrogels made from a novel polymer, oligo(poly(ethylene glycol) fumarate) (OPF), recently developed in our laboratory. Properties of hydrogels made from OPF with initial PEG molecular weights of 860, 3900 and 9300 were examined. The PEG 3900 formulation had a tensile modulus of 23.1 ± 12.4 kPa and percent elongation at fracture of 53.2 ± 13.7%; the PEG 9300 formulation had similar tensile properties (modulus: 16.5 ± 4.6 kPa, elongation: 76.0 ± 26.4%). However, the PEG 860 gels had a significantly higher modulus (89.5 ± 50.7 kPa) and a significantly smaller percent elongation at fracture (30.1 ± 6.4%), when compared with other formulations. Additionally, there were significant differences in percent swelling between each of the formulations. Molecular weight between crosslinks (M_c) and mesh size were calculated for each OPF formulation. M_c increased from 2010 ± 116 g/mol with PEG 860 to 6250 ± 280 g/mol with PEG 9300. Mesh size calculations showed a similar trend (76 ± 2 Å for PEG 860 to 160 ± 6 Å for PEG 9300). It was also found that these hydrogels could be laminated if a second layer was added before the first had completely crosslinked. Mechanical testing of these

laminated gels revealed that the presence of an interfacial area did not significantly alter their tensile properties. These results suggest that the material properties of OPF-based hydrogels can be altered by changing the molecular weight of PEG used in synthesis, and that multi-layered OPF hydrogel constructs can be produced, with each layer having distinct mechanical properties.

**INTRODUCTION**

It has been reported that approximately 25% of all visits to orthopedists are due to knee injuries [70]. Many of these injuries involve articular cartilage, either because of osteoarthritis or traumatic lesions. In many cases, these injuries penetrate through the cartilage and also involve the subchondral bone. Tissue engineering of articular cartilage may help resolve many of the problems associated with repair of osteochondral injuries. In particular, using a combination of a scaffolding material and transplanted cells may result in regeneration of cartilage tissue with the same architecture as native tissue [71, 72]. Articular cartilage is subdivided into several layers, or zones, each with different extracellular matrix compositions and cellular orientation [71]. It is believed that each of these zones plays an important role in articular cartilage function and health of the tissue. For example, the relatively larger amount of collagen in the superficial zone is thought to allow the tissue to withstand shear forces generated at the joint surface [71].

Ideally, in addition to regenerating articular cartilage structure and function, tissue engineering approaches would also reduce patient discomfort and procedure cost by providing minimally invasive options to restore cartilage tissue. For this, an injectable carrier for the transplanted cells would be required. Previous work has shown promising results using poly(ethylene glycol) (PEG) modified with methacrylate end-groups that
can be injected and then crosslinked with ultraviolet light to form a hydrogel.

Chondrocytes embedded in these hydrogels survived the crosslinking process, proliferated and produced cartilage-like extracellular matrix at thickness up to 8 mm [64, 73, 74]. PEG has been found to be minimally toxic in humans and is FDA approved for use in drug manufacture and cosmetic products, making it an excellent choice as a scaffolding material [75, 76]. However, no evidence of degradation has been reported over the course of several weeks in vitro or in vivo for PEG methacrylate-based hydrogels [64, 73, 74]. This could limit the amount of new cartilage tissue that can be formed within these gels. Moreover, to date, no systematic study has been undertaken to determine how material properties of these hydrogels affect the function of cells embedded within them.

Recently, a novel polymer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory (see Figure 3.1). A derivative of PEG, this macromer can be crosslinked through the unsaturated double bond found in the fumarate group. Therefore, this material can be crosslinked in situ with a radical initiator to form a hydrogel without the need for ultraviolet light. In addition, the ester bonds in the fumarate groups are susceptible to hydrolytic cleavage, thus promoting the degradation of these hydrogels [77]. Because of its chemical structure, OPF is also very well-suited to serve as a model system for investigating the effect of changes in material properties on cellular function.

By changing the molecular weight of the PEG used in the formation of this macromer, we hypothesize that the cross-linking density, and therefore the mesh size, of the resulting hydrogel network will be altered. This difference in mesh size will produce different mechanical properties in the various hydrogel formulations. Thus, these studies
were designed to determine the effects of changes in PEG molecular weight on swelling and mechanical properties of crosslinked hydrogels containing OPF.

Additionally, it was found that these hydrogels could be laminated by adding a second layer before the first had completely crosslinked. This could be useful in a clinical setting to physically re-create the zonal organization of articular cartilage by in-situ generation of a multi-phasic cell-polymer construct. In concept, such a construct would be useful only if lamination did not significantly weaken the material. Therefore, a second objective was to determine the effects of lamination on the mechanical properties of a biphasic hydrogel material.

**MATERIALS AND METHODS**

*Synthesis of Oligo(poly(ethylene glycol) fumarate) (OPF)*

Three formulations of OPF were synthesized following the method of Jo et al. [77]. Each formulation contained PEG of different initial number average molecular weight: 9,300 (designated OPF 10K), 3,900 (OPF 4K), and 860 (OPF 1K), as determined by gel permeation chromatography (GPC).

Briefly, PEG, obtained from Aldrich (Milwaukee, WI), was boiled azeotropically in toluene to remove any excess water. The distilled PEG was then dissolved in dichloromethane (distilled before use) (Fisher Scientific, Pittsburgh, PA) or tetrahydrofuran (distilled before use, Aldrich) to produce a 40% (v:v) solution. Appropriate amounts of fumaryl chloride (FuCl, distilled before use) (Acros, Pittsburgh, PA) and triethylamine (TEA, Acros) to obtain a molar ratio of 0.9 PEG:FuCl were added drop-wise over the course of 3-5 hours, while the PEG solution was stirred constantly and held at approximately 0°C by placing the reaction flask in an ice bath. The FuCl and
TEA were diluted at least 1:3 (v:v) in anhydrous dichloromethane or tetrahydrofuran before dripping. The reactions were carried out under nitrogen.

After the addition of the FuCl and TEA was complete, the OPF 10K was removed from the ice bath and allowed to stir for an additional 72 hrs at 25°C, while the OPF 4K and OPF 1K were stirred for an additional 48 hrs at 25°C to assure that the PEG had fully reacted. At this time, the excess dichloromethane was removed by rotary evaporation, and ethyl acetate (Fisher Scientific) was added to the OPF. The solution was then vacuum filtered to remove the salt formed by the reaction of Cl (from FuCl) and TEA. For further purification, the OPF was recrystalized twice in ethyl acetate and then washed three times with ethyl ether (Fisher Scientific). Finally, the resulting powder was vacuum dried at <5 mmHg for approximately 6-8 hrs. The dried product was stored in a sealed container at 4°C until use.

**Gel Permeation Chromatography (GPC)**

The OPF molecular weights were determined via GPC using a system (Waters Model 410, Milford, PA) equipped with a differential refractometer. Both the samples of the PEG used for synthesis and the resulting OPF samples were dissolved in chloroform and filtered (0.45 μm filter) before injection. A Waters column (50-100,000 Da range) was used for these measurements and the GPC was operated at a flow rate of 1 mL/min. Molecular weights were determined from elution time based on a calibration curve generated with PEG standards (Mₙ 550, 1430, 10800, 21200, 59000, Waters). Three samples of each polymer type were tested.

**Nuclear Magnetic Resonance Spectroscopy (NMR)**
$^1$H-NMR spectra were determined using a Bruker Advance 400 MHz NMR system (Bruker Analytik GmbH, Rheinstetten, Germany) operated by a Silicon Graphics O2 workstation (Silicon Graphics, Mountain View, CA). Data were obtained using a 30° pulse angle, 4 s acquisition time and 3 s delay time. Samples were dissolved in CDCl$_3$ with tetramethylsilane (TMS) as the reference (Cambridge Isotope Laboratory, Andover, MA).

**Sample Crosslinking and Lamination**

The OPF formulations were crosslinked in the presence of deionized water using the water-soluble radical initiation system of ammonium persulfate (APS) (EM Science, Gibbstown, NJ) and ascorbic acid (AA) (Sigma, Milwaukee, WI) and poly(ethylene glycol) diacrylate (PEG-DA, $M_n$ 575, Aldrich) as a crosslinker (see Figure 3.1). The weight ratio of OPF to PEG-DA was 2:1. All formulations were 75 weight percent initial H$_2$O, with both the APS and AA at final concentrations of 0.1 M. The solutions were injection molded in rectangular Teflon molds with a thickness of 1 mm. For the non-laminated gels, the molds were then placed in an oven (60° C) for approximately 15 min to expedite crosslinking.

For the laminated hydrogels, the first layer was injected half-way up the Teflon mold and then allowed to partially crosslink at room temperature (10-15 min, depending on the formulation), until the gel was solid but still tacky on top, and the second formulation was injected to fill the mold (see Figure 3.2). The laminated composite was then placed in an oven (60°C) for approximately 15 min to complete the crosslinking process.
Tensile Testing

Sample Preparation

Once removed from the molds, the samples were allowed to swell in distilled, deionized water (ddH₂O) for 12 hrs and then were cut with a metal cutting device into dog-bone shapes. The resulting samples had dimensions of ASTM D638-98 type IV specimens, although the thickness varied depending on the OPF formulation (OPF 1K: 1.05 mm, OPF 4K: 1.45 mm, OPF 10K: 1.70 mm). Five to six specimens of each formulation were tested. Sample types included the three PEG molecular weights with and without lamination (interface between the two layers occurred half-way into the narrow part of the specimen, see Figure 3.2), and samples in which half of the dog-bone was OPF 1K and half was OPF 10K, designated 1K-10K and 10K-1K to indicate the order in which the layers were created. In order to facilitate gripping of the specimens, pieces of balsa wood were glued to the larger portions of the dog-bones using a cyanoacrylate adhesive. This technique is acceptable for tensile testing, as long as the bond between the wood and the hydrogel is stronger than the fracture strength of the hydrogel so there is no slippage at the wood-gel interface [78].

Testing

The hydrogels were subjected to tensile testing as per ASTM D638-98. An Instron testing machine (Model 5565, Canton, MA) with a 50 N load cell was employed for these experiments. OPF 10K samples with and without lamination were tested at a speed of 25 mm/min, while all other samples were tested at 10 mm/min. The specimens were tested at different speeds to assure that they fractured within the time limits set by the ASTM standard (0.5-5 min). Samples were misted with distilled water to maintain
hydration throughout testing. Load-displacement curves were recorded and, knowing the initial cross-sectional area of the thin portion of the samples, this was converted to stress-strain data. From these data, tensile modulus, strength at fracture, percent elongation at fracture, and modulus of toughness were determined. Tensile modulus was calculated as the slope of the linear portion of the stress-strain curve. Percent elongation at fracture was found using

\[ \frac{(L-L_o)}{L_o} \times 100 \]

where \( L_o \) is the original length of the narrow portion of the specimen, and \( L \) is its length just before fracture. Toughness was determined by calculating the area under the stress-strain curve from the initiation of the test until fracture of the sample. For the laminated samples, position of fracture was also recorded.

**Swelling Studies**

**Equilibrium Swelling**

A 24-hr study was performed to determine percent swelling of the various OPF formulations. Samples were injection molded as previously described, and then cut with a cork-borer into discs approximately 10 mm in diameter. Each disc was placed in 200 mL ddH₂O at 25°C for the course of the study. A minimum of three discs of each hydrogel type were weighed at 0, 1, 3, 6, 10 and 24 hrs. In addition, the diameter of each sample was measured with digital calipers (Mitutoyo 500-196CE, Japan) at each time point. After the measurements were taken, the discs were placed back in water until the next time point.
Calculation of Molecular Weight between Crosslinks, Mesh Size and Percent Swelling

Hydrogels were prepared as described above and cut into 16 mm diameter discs before swelling. Determination of the average molecular weight between cross-links, $M_c$, and mesh size, $\xi$, of each hydrogel formulation was based on swelling and mechanical data using theories summarized in Peppas and Barr-Howell [79] and Peppas and Merrill [80], assuming a Gaussian distribution of chain lengths between crosslinks. The equation to determine $M_c$ based on these assumptions when crosslinks have been introduced in the presence of solvent is as follows:

$$\frac{1}{M_c} = \frac{\tau}{\alpha - (1/\alpha^2)} \ast \frac{Q^{1/3}}{R T C_{2,r}} + \frac{2}{\bar{M}_n}$$

where $\bar{M}_n$ is the number average molecular weight of the OPF macromer, $\tau$ is the tensile stress (kPa), $\alpha$ is the elongation, $C_{2,r}$ is the mass concentration of polymer in solution before crosslinking (0.33 kg/L), $R$ is the gas constant (8.31 kPa L/mol K), and $T$ is the temperature at which tensile testing occurred (298 K). $Q$ is the volume swelling ratio of OPF after having reached thermodynamic equilibrium with its solvent and is calculated using

$$Q = (1 - \phi) \frac{V_{2,r}}{V_{2,s}}$$

where $V_{2,s}$ is the polymer volume fraction in the swollen gel and $V_{2,r}$ is the polymer volume fraction in the gel after crosslinking but before swelling. $\phi$ is the sol fraction of the gel.

In order to determine $V_{2,s}$ and $V_{2,r}$, the procedure outlined by Peppas and Barr-Howell was followed using a hanging pan balance apparatus ($n=7$ for each formulation).

$$V_{2,r} = \frac{V_p}{V_{b,r}}$$
\[ V_p = \frac{W_{a,d} - W_{n,d}}{\rho_n} \]

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

where \( W_{a,d} \) is the sample weight in air after cross-linking, \( W_{n,d} \) is the sample weight in a nonsolvent (hexane) after cross-linking, \( W_{a,d} \) is the sample weight in air after swelling in ddH\(_2\)O for 6 hrs and then vacuum drying overnight and \( W_{n,d} \) is the sample weight in hexane after swelling and vacuum drying (\( \rho_n = 0.66 \) g/cm\(^3\) for hexane).

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

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

where \( W_{a,s} \) is the sample weight in air after swelling in ddH\(_2\)O for 6 hrs, and \( W_{n,s} \) is the sample weight in hexane after swelling.

The sol fraction (\( \phi \)) of these hydrogels is estimated using these measurements as:

\[ \phi = \frac{\kappa W_{a,r} - W_{a,d}}{\kappa W_{a,r}} \]

where \( \kappa \) is the weight fraction of the polymer in solution just before crosslinking. For these formulations, \( \kappa = 0.25 \). This estimation represents the theoretical sol fraction based on the fact that the hydrogels were crosslinked with 75 weight percent initial water and assumes that no water was excluded during crosslinking.

From these measurements, mesh size, \( \xi \), can be also calculated as detailed in Peppas and Barr-Howell [79], using

\[ \xi = v_{2,s}^{-13/3} (r_0^{3/2}) \]

From these measurements, mesh size, \( \xi \), can be also calculated as detailed in Peppas and Barr-Howell [79], using

[\( r_0 \) is the initial radius of the hydrogel.]
where $r_{o}^{3}$ is the end-to-end distance of the polymer chains in the unperturbed state. $r_{o}^{3}$ can be determined using $C_{n}$, the characteristic ratio:

$$C_{n} = \frac{r_{o}^{3}}{NI^{2}}$$

where $I = 1.47 \, \text{Å}$, the weighted average of the bond lengths of C-C bonds (1.54 Å) and C-O bonds (1.43 Å). $C_{n}$ is taken to be 4.0 for PEG, as per Billmeyer [81]. $N$ is the number of links between crosslinks and can be calculated based on $M_{c}$. For PEG,

$$N = \frac{3M_{c}}{M_{r}}$$

where $M_{c}$ is the molecular weight of the repeating unit (44 g/mol).

Standard deviations reported for the $M_{c}$ and mesh size calculations were determined using error propagation methods described by Taylor [82].

Swelling data were also used to calculate the swelling ratio for each of the hydrogel formulations using the following formula:

$$(W_{s}-W_{d})/(W_{d}).$$

This parameter provides an indication of how much water (g) is absorbed per 1 g total polymer (OPF + PEG-DA) [77].

**Statistical Analysis**

Results from both the swelling and mechanical studies were compared using ANOVA and Tukey's multiple comparison test ($p<0.05$).

**RESULTS**

**GPC**

GPC analysis indicated that the OPF 1K macromer had an average $M_{n}$ of 4,450 with a polydispersity index (PI) of 2.2, while the corresponding PEG used for macromer
synthesis had an average $M_n$ of 860 with a PI of 1.1. The OPF 4K had an average $M_n$ of 9,240 with PI = 1.9. The PEG used to produce this macromer had an average $M_n$ of 3,900 with a PI of 1.1. The OPF 10K formulation had an average $M_n$ of 13,950 and PI of 1.4, and its corresponding PEG had an average $M_n$ of 9,300 and PI = 1.1.

**NMR**

NMR results for each type of OPF confirmed presence of peaks at 6.8 ppm (data not shown), which are attributed to hydrogen atoms associated with the unsaturated double bond in the fumarate group [77].

**Tensile Testing**

Tensile testing revealed that the modulus of the OPF 1K formulation (89.5 ± 50.7 kPa) was significantly higher than the other two sample types (OPF 4K: 23.1 ± 12.4 kPa, OPF 10K: 16.5 ± 4.6 kPa) (see Figure 3.3). PEG molecular weight did not have a significant effect on strength at fracture (10-25 kPa for all samples, see Figure 3.4) or toughness (3-6 kPa for all samples, see Figure 3.5). However, the OPF 1K had a significantly smaller percent elongation at fracture (30.1 ± 6.4%) than the OPF 10K hydrogel (76.0 ± 26.4%) (see Figure 3.6).

When compared with non-laminated gels of the same PEG molecular weight, the presence of an interface did not have a significant effect on any of the mechanical properties tested. In all cases, the samples broke in the thin portion of the sample, but, upon visual inspection, the line of fracture did not follow the line of lamination. For the 10K-1K and 1K-10K samples, the only calculation that could be completed was percent elongation at fracture due to varying cross-sectional area within the samples. The addition of the 1K phase in the 1K-10K and 10K-1K samples significantly decreased the
percent elongation at fracture (29.1 ± 5.1% and 29.9 ± 7.4%, respectively) when compared with the non-laminated OPF 10K samples (76.0 ± 26.4%) (see Figure 3.6). The order in which the layers were added did not have a significant effect on the percent elongation at fracture (Figure 3.6). Additionally, in 12 samples tested (6 1K-10K, 6 10K-1K), there seemed to be no trend in where (which layer) fracture occurred.

Swelling Studies

Molecular Weight between Crosslinks, Mesh Size and Sol Fraction Calculations

Results of $M_c$, mesh size and sol fraction calculations for each OPF formulation are found in Table 3.1. These calculations indicate that $M_c$ and mesh size increased as the initial PEG molecular weight in the OPF formulations increased. The sol fraction of the OPF 10K samples was significantly lower than that of the other formulations, while the sol fraction of the OPF 4K hydrogels was significantly higher than for the OPF 1K.

Swelling Ratio

Swelling studies indicated that equilibrium swelling was reached within 1 hr. The percent increase in diameter (from the initial crosslinked state to the swollen state) for various formulations are included in Table 3.1. Figure 3.7 depicts the swelling ratio of the different hydrogels at 6 hrs. Statistical analysis revealed that, at 6 hrs, the OPF 1K formulation had a significantly lower swelling ratio (8.6 ± 0.3) than either the OPF 4K (14.8 ± 0.7) or the OPF 10K (15.7 ± 0.5). In addition, the OPF 4K hydrogels swelled significantly less than the OPF 10K samples.

Discussion

These experiments were designed to determine the effect of PEG molecular weight on the swelling and mechanical properties of hydrogels made from a novel PEG
derivative, OPF, recently developed in our laboratory. In addition, it was found that OPF hydrogels could be laminated if a second layer was added before the first had completely crosslinked (see Figure 3.8). Therefore, the effect of an area of interface on tensile properties of these laminated hydrogels was also studied.

In order to create hydrogels with different mesh sizes, OPF was synthesized using PEG of molecular weights 860, 3900 and 9300. Because of the structure of OPF (see Figure 3.1), the spacing of unsaturated fumarate double bond, through which crosslinking occurs, is determined by the length of the PEG chain used in synthesis. Therefore, by choosing PEG with various molecular weights spanning an order of magnitude, it was believed that the resulting gels would have differing mesh sizes after crosslinking.

After synthesis of each of the formulations, GPC and NMR analysis showed that the PEG had been converted to OPF. However, as seen by Jo et al. [77], GPC results (not shown) confirmed a decrease in the number of PEG chains incorporated in the macromer and a relative increase in the amount of free (unreacted) PEG as the PEG molecular weight increased. This may be due to steric considerations in the larger PEG molecules, which hinder the addition of the fumarate groups to the ends of the molecule.

**Non-laminated Hydrogels**

Hydrogels were formed from the OPF using PEG-DA as a crosslinking agent and the radical initiation system AA/APS, as shown in Figure 3.1. This redox pair initiator system was chosen because it is water-soluble so no organic solvents, which may harm cells *in vivo*, were required for crosslinking; however, *in vivo* biocompatibility of this initiator system still needs to be assessed. PEG-DA was employed to reduce the crosslinking time and increase the strength of the crosslinked hydrogels. In this study,
hydrogels were placed in an oven (60°C) to expedite crosslinking. Crosslinking times at 37°C were not quantified in these experiments as this was not an objective of the study. Therefore, these hydrogels may need to be reformulated to obtain optimal crosslinking times before use in vivo.

Because weight ratios were used (2 OPF:1 PEG-DA by weight), and due to the varying molecular weights of the component materials, the ratio of fumarate double bonds: acrylate double bonds was different for each formulation. For the OPF 10K and OPF 4K, this ratio was small (0.04 and 0.06, respectively), while in the OPF 1K formulation the ratio was an order of magnitude larger (0.5). This may indicate that, in the OPF 10K and OPF 4K hydrogels, the acrylate groups would have more opportunity to react with other acrylate groups, rather than crosslinking between fumarate double bonds. While the inconsistencies in double bond ratio may have an effect on mesh size calculations, the intent of this study was to choose formulations that would result in varied mechanical and swelling properties, so we believe that these differences are not a serious concern for these experiments.

Although these hydrogels are intended for eventual use in sites that must withstand compressive forces, such as the knee, tensile testing was chosen in this study to examine the fracture properties of both laminated and non-laminated hydrogels. In particular, experiments were designed to determine if delamination occurred before fracture. Additionally, tensile data have been used as the basis for calculations of $M_c$ and mesh size in hydrogels [79, 83].

The modulus, fracture strength, toughness and percent elongation at fracture were determined from the tensile data. Modulus is a measure of material stiffness, while
toughness indicates the amount of energy a material can absorb before fracturing. As expected for a material with shorter chains between crosslinks, the OPF 1K was found to have a significantly higher modulus than the other two formulations, and a significantly smaller elongation at fracture. It was thought that the OPF 1K hydrogels may also have a higher fracture strength due to the greater possibility of crosslinking per volume, but this was not found to be the case. All formulations also had similar toughness. However, these results do indicate important material properties, such as stiffness, can be controlled by changing the molecular weight of the PEG in the OPF.

In swelling studies, a significant difference in weight percent swelling was seen for all three OPF formulations. The theoretical sol fraction calculations, determined based on the assumption that the hydrogels contained 75 weight percent water immediately after crosslinking, indicated a lower sol fraction for the OPF 10K than the other formulations. This may be because for the same weight ratio of OPF:PEG-DA the number of fumarate double bonds per chain decreases, so that the probability of a chain being part of the network increases. These results show that it is possible to alter the swelling properties of OPF hydrogels by varying the PEG length in the macromer. Taking the mechanical and swelling data together, the OPF 1K hydrogels are stiffer, elongate less and swell less than hydrogels made with the other formulations. These are all indications that the OPF 1K results in a hydrogel with a smaller $M_c$ and mesh size than the other formulations. While no significant difference was observed between the mechanical properties of OPF 4K and OPF 10K hydrogels, there was a significant difference in swelling.
When using the tensile data to calculate mesh size and $M_c$, it should be noted that the OPF 10K samples were tested at approximately twice the speed of the other samples (25 mm/min vs. 10 mm/min). This was done to insure that fracture occurred within the time limits specified by the ASTM standards. Rates of approximately 25 mm/min have been used previously in tensile testing of hydrogels to determine $M_c$ [80]. For all formulations, fracture occurred within 2.5 minutes and the samples were continuously misted with distilled water during testing, so dehydation of the gels, which can affect the measured tensile properties, was minimal.

Calculations based on this data show that the $M_c$ varies from $2010 \pm 116$ g/mol (OPF 1K) to $6250 \pm 2806$ g/mol (OPF 10K), with corresponding mesh sizes from $76 \pm 2$ Å to $160 \pm 6$ Å (Table 3.1). The equation used for the calculations is based on rubber elasticity theory [84]. Most hydrogels, when swollen, behave as rubbers [83], so these equations, with modification for initial polymer concentration before crosslinking, can be used. Development of this theory assumes affine deformation and Gaussian chain-length distribution between crosslinks [85, 86]. These assumptions are generally valid for moderately-swollen hydrogels. Such hydrogels usually have a volumetric swelling ratio (Q) of 1.5-5 [79]. However, when hydrogels are highly swollen, the finite extensibility of the chains results in deviations from Gaussian behavior [85-87]. The volumetric swelling ratio for the OPF hydrogels range from 1.23 to 3.02 (Table 3.1), so the use of rubber elasticity equations is valid for these gels. However, these equations were developed for systems in which the sol fraction of the crosslinked network was negligible [79]. In this case, the sol fractions of the three OPF formulations are substantial (17% - 35%), which led to modification of the expression for $M_c$ to include a sol fraction term. In addition,
this model also assumes the length of the crosslinking agent is negligible compared to that of the polymer backbone [88]. A model addressing properties of the crosslinking agent would provide more accurate estimates of $M_c$/mesh size in these hydrogels, but the swelling and mechanical data strongly suggest that there are differences in mesh size between the three formulations. This work provides a method to synthesize hydrogels with controlled mesh size and investigate the effects of hydrogel material properties on chondrocyte function and cartilage tissue formation, currently in progress in our laboratory.

*Laminated Hydrogels*

Tensile testing revealed that the presence of an interface in the laminated gels does not significantly affect any of the values calculated. Additionally, the line of fracture in each sample did not seem to follow the line of lamination when inspected after testing. This indicates that the layers are well-bonded and that the interfacial area does not provide an intrinsic point of weakness in the material, which is important if laminated gels of this type are to be utilized *in vivo* in load-bearing sites.

The presence of two phases with different mechanical properties did affect the overall tensile properties of the biphasic hydrogels. The percent elongation at fracture was significantly reduced when the OPF 1K phase was added to the OPF 10K, as compared with the non-laminated OPF 10K hydrogel. It was hypothesized that, in the case when the OPF 10K is added as the second layer, the smaller mesh size of the OPF 1K hydrogel may hinder the ability of the OPF 10K gel to penetrate into the already existing network and thus form a strong interface, but it was found that the order in which
the OPF 10K formulation was incorporated into the gel had no effect on percent elongation at fracture, or in which layer fracture occurred.

When developing this system for use in tissue engineering applications, one must carefully choose the swelling and mechanical properties of the various phases so that they are compatible. As noted in Table 3.1, at equilibrium swelling, the diameters of the sample discs, each originally 10 mm, increased by 33% (OPF 1K) - 60% (OPF 10K). Such differences in swelling, seen also in the weight percent swelling and Q differences between the various formulations, cause stresses to be developed between layers if two materials with very different swelling properties are used. When laminated constructs like those shown in Figure 3.8, but with diameters greater than approximately 10 mm, were created, the constructs became curved as equilibrium swelling was reached. In some cases, the forces generated caused the OPF 1K phase to tear in half, perpendicular to the line of lamination. While a combination of these two particular OPF formulations would be undesirable for use in vivo due to the possibility of ripping, this phenomenon provides further evidence that the interface between the layers is strong, as the tear did not propagate along the line of lamination, but rather perpendicular to it.

CONCLUSIONS

The molecular structure of the novel macromer OPF, a derivative of PEG that can be crosslinked to form hydrogels, allows for control of mesh size by alteration of the initial PEG molecular weight used in synthesis. In this study, three types of OPF were used to form hydrogels with different mechanical and swelling properties. The OPF 1K hydrogel, made from the smallest PEG, swelled less, was stiffer, and elongated less before fracture when compared to the other hydrogel types. These hydrogels can also be
combined in layers to form biphasic gels, with each phase having different material properties. The presence of an interface in the laminated hydrogels did not have a significant effect on mechanical properties for any of the OPF types, indicating that the interface did not produce an area of weakness in the resulting composite gels.
**TABLE 3.1:** Results of swelling studies for OPF hydrogels after 6 hrs in ddH₂O. Data are presented as means ± standard deviation (n=3 minimum).

<table>
<thead>
<tr>
<th>OPF Formulation</th>
<th>Increase in Diameter (%)</th>
<th>Volumetric Swelling Ratio (Q)</th>
<th>Sol Fraction (ϕ, %)</th>
<th>Molecular Weight between Crosslinks (Mₓ, g/mol)</th>
<th>Mesh Size (ξ, Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPF 1K</td>
<td>33 ± 4</td>
<td>1.23 ± 0.06</td>
<td>30 ± 2</td>
<td>2010 ± 116</td>
<td>76 ± 2</td>
</tr>
<tr>
<td>OPF 4K</td>
<td>52 ± 6</td>
<td>1.73 ± 0.16</td>
<td>35 ± 4</td>
<td>4300 ± 200</td>
<td>125 ± 7</td>
</tr>
<tr>
<td>OPF 10K</td>
<td>60 ± 2</td>
<td>3.02 ± 0.12</td>
<td>17 ± 2</td>
<td>6250 ± 280</td>
<td>160 ± 6</td>
</tr>
</tbody>
</table>
**FIGURE 3.1:** Schematic of crosslinking reaction that occurs with OPF and PEG-DA using the water soluble initiator system APS/AA. Alterations in the molecular weight of PEG in the OPF (represented by n in diagram) affect the mesh size of the resulting crosslinked network.
**FIGURE 3.2:** Steps in formation of laminated hydrogels for tensile testing. Grey and white shading represent different OPF formulations.
**Figure 3.3:** Tensile modulus of OPF hydrogels of various PEG molecular weights with and without the presence of an interface (±SD, n=5). The symbol * indicates OPF 1K (no interface) had a significantly higher modulus than the other non-laminated formulations (p< 0.05).
**Figure 3.4:** Strength at fracture of OPF hydrogels of various PEG molecular weights with and without the presence of an interface (±SD, n=5).
**Figure 3.5:** Toughness of OPF hydrogels of various PEG molecular weights with and without the presence of an interface (±SD, n=5).
**Figure 3.6:** Percent elongation at fracture of OPF hydrogels with and without the presence of an interface (±SD, n=5-6). The symbol * indicates non-laminated OPF 10K hydrogels had a significantly higher elongation at fracture than the OPF 1K (non-laminated) or the biphasic hydrogels (10K/1K and 1K/10K).
**Figure 3.7:** Swelling ratio of OPF hydrogels at 6 hrs (±SD, n=7). The symbol * indicates there was significant differences in swelling between all three hydrogel formulations (p<0.05).
**Figure 3.8:** Image of biphasic composite hydrogels before and after swelling in ddH$_2$O overnight. Top layer: OPF 10K, bottom layer: OPF 1K.
CHAPTER IV

EFFECT OF DRYING HISTORY ON SWELLING PROPERTIES AND CELL ATTACHMENT TO Oligo(Poly(ETHYLENE GLYCOL) FUMARATE) HYDROGELS

ABSTRACT

In these experiments, the effects of the drying history of hydrogels made from a novel polymer, oligo(poly(ethylene glycol) fumarate) (OPF) with two different poly(ethylene glycol) (PEG) molecular weights (approximately 920 (1K) and 9,110 (10K) g/mol) were investigated. The hydrogels were either formed, dried and then swelled, representing what may occur in the case of a pre-formed membrane for guided tissue regeneration, or were formed and swelled immediately, as may occur with an injectable material for such applications. Subsequently, swelling properties, sol fraction, and polymer network structure (as indicated by differential scanning calorimetry), as well as attachment of human dermal fibroblasts to these hydrogels at 4 and 24 hours was examined.

It was found that drying before swelling caused a significant reduction in final fold swelling of OPF hydrogels, regardless of OPF formulation or method of drying (air-dried or vacuum-dried) (e.g. PEG 10K swollen first: 13.94±0.35 vs. vacuum first: 6.53±0.12; PEG 1K swollen first: 8.99±0.47 vs. vacuum first: 2.26±0.08). This decreased swelling correlated to significantly higher cell attachment (% seeded) to these hydrogels at 24 hrs (PEG 10K vacuum first: 21.1±4.7% vs. swollen first: 7.1± 5.5%; PEG 1K vacuum first: 58.2±2% vs. swollen first: 7.4±2.2%). LIVE/DEAD staining

followed by microscopic analysis revealed attached cells were viable, yet rounded, and that, in the case of the PEG 1K dried-first samples, undulations in the surface visible in the hydrated state may have affected cell adhesion. Regardless of treatment, all hydrogels showed significantly less cell attachment than the tissue culture polystyrene control after 24 hrs (104.9±4.4%). These results suggest that, by altering the PEG molecular weight used in synthesis, OPF hydrogels may be tailored to produce desired swelling properties and reduce non-specific cell adhesion for either injectable or pre-formed applications, thus providing a potential alternative material for use in guided tissue regeneration procedures.

INTRODUCTION

Guided tissue regeneration (GTR) is based on the principle that various cell types migrate into tissue defects at different rates. Therefore, through correct placement of a barrier membrane, it is possible to prevent the entry of unwanted tissue types (usually fibrous) while encouraging proliferation and matrix production from certain cells (usually osseous) populating the defect [89]. GTR has been employed successfully in human and animal trials for a variety of dental applications, often with the goal of aiding integration of implants with surrounding tissue [89-96]. This approach has also shown promise in regeneration of cranial [97-102] and long-bone defects [103-106].

The requirements of barrier membranes for GTR include that they be biocompatible, occlude ingrowth of unwanted cell types, and maintain space for generation of new tissue [107-109]. The most widely studied non-biodegradable material for this application is expanded poly(tetrafluoroethylene) (e-PTFE or Gore-Tex®), which has shown to be efficacious in promoting bone formation in both animal and clinical
studies [90, 91, 95, 96, 107-111]. However, the risk of infection with these semi-permanent membranes and the need for a second surgery to remove them after tissue regeneration is complete have led investigators to study biodegradable alternatives, the most common of which include the natural-based material collagen or the synthetic-based materials poly(glycolic acid) or poly(lactic acid) [108]. All of these have recently been explored for GTR in both animal and clinical trials [92-94, 108, 110].

In addition to being biodegradable, the ideal GTR membrane would improve bone formation within the defect [97, 107]. This could be achieved by using the membrane material as a carrier for soluble or insoluble factors that encourage bone tissue growth [90, 112].

A final consideration for GTR membranes, as with all tissue-engineering products, is ease of handling and use in a clinical setting [107, 109]. In some cases, a pre-formed barrier membrane such as those currently employed may be required, while for other defects an injectable material may be more effective. In either case, appropriate material properties (e.g. swelling and mechanical properties) in vivo are essential to the success of the therapy [109, 110].

Our laboratory has recently developed a novel hydrogel system based on oligo(poly(ethylene glycol) fumarate) (OPF), that is being investigated as a biodegradable material to guide bone tissue ingrowth [113]. In previous studies, after leaching and pre-sterilization, this hydrogel material elicited a mild tissue response when implanted in a rabbit tooth socket model [114], subcutaneously in rabbits, or in sub-critical-sized rabbit cranial defects [115]. In addition to its biocompatibility, OPF offers several advantages towards development of the ideal GTR membrane. The ester bonds in
the fumarate moieties are susceptible to hydrolytic cleavage, thus allowing for the
degradation of OPF hydrogels \textit{in situ} to form space for new tissue. Prior work has
demonstrated that non-specific cell adhesion to OPF hydrogels is minimal, and that cell
attachment of marrow stromal osteoblasts can be modulated by the addition of Arg-Gly-
Asp (RGD) peptide sequences to these gels [113]. Through the inclusion of such
insoluble signals, OPF-based materials could encourage bone formation in specific
locations while preventing ingress of other cell types.

The system's versatility allows for its use as either an \textit{in situ} polymerizable or
pre-formed scaffold [113, 116]. When crosslinked \textit{in situ}, the hydrogel will swell
immediately, while if it is pre-formed, it may be dried before implantation and eventual
swelling. Characterization of the swelling properties of the OPF hydrogels is important
for their final clinical applications. Additionally, changes in swelling may affect non-
specific cell adhesion, which should be minimal for successful GTR materials.

Therefore, these studies were designed to elucidate the effects of drying history on
swelling, sol fraction, and polymer network structure (as indicated by differential
scanning calorimetry) of OPF hydrogels with two different poly(ethylene glycol) (PEG)
molecular weights. A second objective was to determine if drying history of hydrogels
made from these OPF formulations resulted in differences in adhesion of human dermal
fibroblasts after 4 and 24 hrs.
MATERIALS AND METHODS

Oligo(poly(ethylene glycol) fumarate) (OPF) Synthesis and Characterization

Synthesis

Two formulations of OPF were synthesized as described in Jo et al. [77]. The formulations contained PEG of different initial number average molecular weight: ~9,110 g/mol (designated OPF 10K) or ~920 g/mol (OPF 1K), as determined by gel permeation chromatography (GPC).

Briefly, PEG, obtained from Aldrich (Milwaukee, WI), was distilled and dissolved in dichloromethane (distilled before use) (Fisher Scientific, Pittsburgh, PA) to produce a 40% (v:v) solution. Appropriate amounts of fumaryl chloride (FuCl, distilled before use) (Acros, Pittsburgh, PA) and triethylamine (TEA, Acros) to obtain a molar ratio of 0.9 PEG:FuCl were added drop-wise to the PEG solution, held at approximately 0°C. The reactions were carried out under nitrogen.

After addition of the FuCl and TEA, the OPF formulations were allowed to stir for an additional 24-72 hrs at 25°C to assure completion of the reaction. At this time, the excess dichloromethane was evaporated, and, after removal of the Cl-TEA salt, the OPF was recrystallized twice in ethyl acetate (Fisher Scientific) and then washed three times with ethyl ether (Fisher Scientific). Finally, the resulting powder was vacuum dried at <5 torr and stored in a sealed container at 4°C until use.

Gel Permeation Chromatography (GPC) and Nuclear Magnetic Resonance Spectroscopy (NMR)

After synthesis, the OPF was characterized via GPC and NMR. A GPC system (Waters Model 410, Milford, PA) equipped with a differential refractometer was used to
determine molecular weight of both the PEG used for synthesis and the resulting OPF. After dissolution in chloroform and pre-filtering (0.45 μm filter), samples were injected into a column (50-100,000 g/mol range, Waters) at a flow rate of 1 mL/min. Molecular weights were determined from elution time based on a calibration curve generated with PEG standards (M_n 550, 1430, 10800, 21200 g/mol, Waters). Three samples of each material were evaluated. In order to confirm addition of the fumarate units to the OPF, samples were dissolved in CDCl_3 (tetramethylsilane (TMS) reference; Cambridge Isotope Laboratory, Andover, MA) and ^1^H-NMR spectra were recorded with a Bruker Advance 400 MHz NMR system (Bruker Analytik GmbH, Rheinstetten, Germany) operated by a Silicon Graphics O2 workstation (Silicon Graphics, Mountain View, CA).

**Swelling Studies**

**Sample Preparation**

OPF was crosslinked in the presence of deionized water using the water-soluble redox radical initiation system of ammonium persulfate (APS) (EM Science, Gibbstown, NJ) and ascorbic acid (AA) (Sigma, Milwaukee, WI). Poly(ethylene glycol) diacrylate (PEG-DA, M_n 575, Aldrich) was included as a crosslinking agent (2 OPF: 1 PEG-DA by weight) [116]. The formulations were 75 weight percent initial H_2O, with both the APS and AA at final concentrations of 0.1 M. After mixing, the solutions were injected into Teflon molds with a thickness of 1 mm and then placed at 60° C for approximately 15 min to expedite crosslinking.

After crosslinking, the hydrogels were cut into discs with a cork-borer (13 mm diameter, 1 mm thickness). These discs were either dried overnight at <1 torr and then swollen for 16 hours to equilibrium swelling in distilled, deionized water at 25°C (VD-
ES), dried overnight at 760 torr, then dried for an additional 12 hours at <1 torr and then swollen (AD-ES), or swollen first and then dried at either <1 torr or 760 torr (ES-VD or ES-AD). This experiment is depicted as "Cycle 1" in Figure 4.1. Fold swelling and sol fraction were calculated for each treatment in this first swelling study (n = 4-5 per treatment), and were also calculated for each cycle of a second swelling study in which the swelling/drying treatments were repeated 3-4 times on the same samples (see Figure 4.1; n = 4-5 per treatment). In the second study, drying was completed via vacuum only. In addition, in the second study, the wet weights between samples that had been dried immediately after fabrication and then swollen (S1) with those that had been swollen after fabrication, dried and then swollen again (S2') were compared.

**Calculation of Fold Swelling and Sol Fraction**

In both swelling studies, fold swelling and sol fraction for the different drying histories were determined using the following equations:

Fold Swelling: \[ \frac{W_s - W_d}{W_d} \]

Where \( W_s \) is the swollen weight of the sample for cycle i (e.g. Si or Si' in Figure 4.1) and \( W_d \) is the dry weight following swelling for that cycle (e.g. Di or Di' in Figure 4.1).

Sol Fraction: \[ \frac{W_{do} - W_{du}}{W_{do}} \]

Where \( W_{do} \) is the sample's dry weight before swelling and \( W_{du} \) is the dried weight after swelling for each cycle. In the case of the repeated swelling/drying study, the sol fraction was calculated with reference to the original dry weight before swelling (D0 in Figure 4.1). For both swelling studies, the average \( W_{do} \) from the corresponding dried-first samples was used to calculate sol fraction for each type of swollen-first samples.
**Differential Scanning Calorimetry (DSC)**

To further explain data obtained in swelling experiments, DSC (heating rate 10°C/min, Model 2920, TA Instruments, New Castle, DE) was performed on both directly swollen (ES) and VD-ES samples in the hydrated state (n = 3 per treatment). Evidence of phase transitions was evaluated by examining heat flow (W/g) vs. temperature (°C) graphs in a range from 30°C to 120°C. Average melting points for dry, linear OPF (not crosslinked) were similarly determined (n = 3 per formulation).

**Cell Attachment Studies**

To determine cell attachment to the hydrogels, OPF squares (1 mm thick) were fabricated as described above, but with phosphate-buffered saline (PBS, Gibco, Grand Island, NY) instead of deionized water. Subsequently, the hydrogels were either swollen for 16 hours to equilibrium swelling in PBS (ES) or dried overnight at <1 torr and then swollen for 16 hours to equilibrium swelling (VD-ES). After swelling, 21 mm diameter discs were cut from the squares with a cork-borer. For sterilization, the discs were placed in PBS and exposed to UV light for 3 hr. Following sterilization, the samples were placed in 12-well tissue culture plates and weighted down with sterile steel annuli (inner diameter 15.5 mm).

Human dermal fibroblasts (HDFs, passage 17, Clonetics, San Diego, CA) were seeded at a concentration of 20,000 cells/cm² on the OPF discs and on control tissue culture polystyrene (TCPS) wells. The total volume of media and number of cells/well were scaled to maintain the above seeding density and height of media in the wells between wells with steel annuli (experimental) and those without annuli (control wells). At 4 and 24 hours, the samples were rinsed once with PBS, transferred to a clean well-
plate, and then treated with trypsin/EDTA (0.05\% trypsin/0.53 mM EDTA, Gibco, Grand Island, NY). Control wells were also rinsed once with PBS and trypsinized. The number of cells released from the sample surface was counted with a cell-size analyzer (Beckman-Coulter, Fullerton, CA) and the fraction of seeded cells that attached was determined for each sample type (n = 4):

\[
\text{Fraction of attached cells: } \frac{C_f}{C_0}
\]

Where \(C_f\) is the number of cells released from the surface of the hydrogel with trypsin at either 4 or 24 hrs after seeding and \(C_0\) is the original number of cells added to the well.

In addition, at 24 hours, cells were stained with LIVE/DEAD reagent (final dye concentration 2 mM calcein AM, 4 mM ethidium homodimer-1, Molecular Probes, Eugene, OR) and visualized with a fluorescence microscope (Eclipse E600 with Y-FL epi-fluorescent attachment and triple (DAPI, FITC and Texas Red) filter set, Nikon, Melville, NY) and attached video camera (3CCD Color Video Camera DXC-950P, Sony, Park Ridge, NJ).

**Statistical Analysis**

Data from all studies were analyzed using ANOVA and Tukey’s Multiple Comparison Test or an unpaired Student’s t-Test (p ≤ 0.05).

**RESULTS**

**OPF Synthesis and Characterization**

GPC analysis indicated that the OPF 1K synthesized had an average \(M_n\) of 5,450 with a polydispersity index (PI) of 1.8, while the corresponding PEG used for macromer synthesis had an average \(M_n\) of 920 with a PI of 1.1. The OPF 10K had an average \(M_n\) of
12,500 with a PI of 1.3. The PEG used to produce this macromer had an average $M_n$ of 9,110 with a PI of 1.2. NMR results for each type of OPF confirmed the appearance of peaks at 6.8 ppm (data not shown), indicating the presence of fumarate groups within the molecules [77].

**Swelling Studies**

**First Swelling Study: Comparison of Drying Methods**

**Fold Swelling**

As detailed in Table 4.1, significantly higher swelling was found with the OPF 10K than with the OPF 1K samples, regardless of whether the hydrogels were swollen or dried first, or the drying method employed. Comparing only the 10K specimens, the gels that were swollen first demonstrated a significantly higher fold swelling than those dried first. There was no difference in fold swelling between samples that were air-dried or vacuum-dried.

Within the 1K samples, it was found that gels that were swollen first showed a significantly higher fold swelling than gels that were dried first. In addition, the OPF 1K ES-VD samples demonstrated a significantly higher fold swelling than the ES-AD treatment. Inversely, the gels that were vacuum-dried before swelling (VD-ES) had significantly less swelling than the AD-ES samples.

**Sol Fraction**

Results of sol fraction calculations (Table 4.1) indicate that when the hydrogels were dried first, there was a significantly higher sol fraction with the OPF 10K than OPF 1K samples, regardless of drying method. Within the OPF 10K samples, the AD-ES hydrogels had a significantly greater sol fraction than the other treatments, which were all
statistically similar. In the OPF 1K specimens, there was a larger sol fraction with gels that had been swollen first than those that had been dried first, while there was no statistical difference between samples that were air-dried vs. vacuum-dried.

**Second Swelling Study: Repeated Swelling/Drying**

**Fold Swelling**

In this study (see Figure 4.2 A for summary of results), swelling of samples of the same composition were compared through several swelling/drying cycles. For the OPF 10K hydrogels that were swollen first, it was found that there was significantly higher fold swelling in the first cycle (14.76±0.16) than the other three (Cycle 2: 6.16±1.07, Cycle 3: 5.80±1.23, Cycle 4: 5.22±0.45) and that there was no significant difference in swelling between the last three cycles. The 10K hydrogels that were dried first demonstrated significantly less swelling with each cycle (Cycle 1 fold swelling: 6.98±0.16, Cycle 2: 4.80±0.16, Cycle 3: 4.38±0.13).

In the OPF 1K samples that were swollen first, the first cycle resulted in a significantly higher fold swelling (6.37±0.35) than the rest of the cycles (Cycle 2: 3.63±0.22, Cycle 3: 3.17±0.17, Cycle 4: 2.85±0.26) and there was no significant difference in swelling between the last three cycles. For the 1K specimens that were dried first, statistically equivalent swelling for all cycles was found (Cycle 1 fold swelling: 3.42±0.24, Cycle 2: 3.45±0.15, Cycle 3: 3.23±0.15).

Comparing between samples after the first swelling/drying cycle, the OPF 10K showed significantly higher swelling than the OPF 1K hydrogels. It was also found that the swollen-first gels swelled more than the dried-first samples, regardless of OPF molecular weight.
Comparison of Wet Weights

No significant difference between the wet weights of S1 and S2' samples made from OPF 10K was found (S1: 0.22±0.01 g, S2': 0.22±0.04 g). Similarly, there was no significant difference evident with the 1K formulation (S1: 0.19±0.01 g, S2': 0.18±0.01 g).

Sol Fraction

As well as swelling ratio, sol fraction was calculated for these samples through several swelling/drying cycles (Figure 4.2 B). It was found that there was no significant difference in sol fraction for each treatment between cycles, irrespective of OPF molecular weight or whether the gels had been swollen or dried first (e.g. 10K ES-VD Cycle 1: 0.38±0.04, Cycle 2: 0.40±0.03, Cycle 3: 0.40±0.04, Cycle 4: 0.40±0.04; 1K VD-ES Cycle 1: 0.15±0.01, Cycle 2: 0.15±0.01, Cycle 3: 0.15±0.01). Statistical analysis revealed that, after the first cycle, the OPF 1K swollen-first specimens had larger sol fractions than their dried-first counterparts, while the inverse was true for the OPF 10K gels. In addition, both types of OPF 10K hydrogels had greater sol fractions than the OPF 1K samples (10K ES-VD Cycle 1: 0.38±0.04, 1K ES-VD: 0.26±0.02, 10K VD-ES: 0.47±0.01, 1K VD-ES: 0.15±0.01).

DSC

After examination of endotherms on the heat flow (W/g) vs. temperature (°C) graphs recorded by the DSC, the average melting points of dry, linear 10K and 1K OPF were determined to be 60.4±0.8°C and 39.4±0.9°C, respectively. No evidence of endotherms was seen for any of the hydrated hydrogel samples at temperatures below 98°C (see Figure 4.3 for representative graphs).
Cell Attachment Studies

Results of cell attachment studies at 4 and 24 hours are summarized in Figure 4.4. At 4 hours, there was a significantly higher percent attachment of HDFs to TCPS control wells (91±2.6%) than to any of the hydrogel samples. Within the OPF samples, the 1K VD-ES showed significantly higher attachment (40.4±11.7%) than the other treatments, which were statistically similar (10K ES: 20.3±7.8%, 10K ES-VD: 7.9±7.1%, 1K ES: 6.5±1.1%).

After 24 hours, significantly fewer cells were found on the OPF samples than on TCPS, which showed nearly complete attachment of seeded cells (104.9±4.4% attachment). As at 4 hours, the 1K VD-ES samples demonstrated significantly higher HDF attachment (58.2±2%) than the remainder of the hydrogel samples. Also at this time point, the 10K VD-ES exhibited significantly more attached cells (21.1±4.7%) than the OPF samples that had been swollen directly after fabrication (10K ES: 7.1±5.5%, 1K ES: 7.4±2.2%).

When comparing attachment for the same sample type over time, there were significantly more cells found on the 10K ES specimens at 4 hours than at 24 hours. The opposite was true for the OPF samples that were dried before swelling, as well as the TCPS control. There was no significant difference in attachment to the 1K ES samples between the two time points.

After 24 hrs, the majority of fibroblasts attached to the OPF and treated with LIVE/DEAD reagent fluoresced green, indicating viability (Figure 4.5). However, the cells had a rounded morphology on all samples examined. In the case of the 1K VD-ES
hydrogels, microscopic evaluation also revealed the presence of undulations on the hydrogel surface that were not seen with the other sample formulations.

**DISCUSSION**

After completion of the synthesis, NMR and GPC analysis confirmed the presence of OPF macromers. As shown previously in our laboratory, OPF hydrogels with varied swelling and mechanical properties can be created by altering the PEG molecular weight used in synthesis of the oligomer [77, 116]. Therefore, in this study, PEG chains with initial molecular weights of approximately 1K and 10K were chosen to determine the effects of drying history on swelling and cell attachment to OPF hydrogels with a range of material properties.

From both swelling studies (Table 4.1 and Figure 4.2), OPF 10K hydrogels showed greater swelling than 1K hydrogels, regardless of drying history. In addition, if the gels were swollen immediately after crosslinking, they demonstrated significantly larger swelling than those dried after fabrication, irrespective of the OPF formulation or drying method. In the first swelling study, within the OPF 10K samples, there was no difference in swelling between air-dried and vacuum-dried gels. For the OPF 1K, ES-VD samples demonstrated a significantly higher fold swelling than the ES-AD treatment, while the opposite was found for the specimens that were dried first (VD-ES < AD-ES). The sol fractions from the first study (Table 4.1) also revealed no clear trend due to vacuum vs. air-drying. These results indicate that the method of drying did not impact the swelling properties as drastically as whether the gels were swollen or dried immediately after fabrication. Therefore, for the remainder of the experiments, only the vacuum-drying method was employed.
In order to explore the underlying reasons for the marked differences in fold swelling between hydrogels that had been dried prior to swelling and those that had not, further analysis was performed. Reduced final fold swelling in the dried-first samples could be caused by additional crosslinking due to the continued presence of initiator in the dried state, the formation of crystalline phases in the polymer during drying [117, 118] increased entanglements as water is removed and the polymer chains come into closer contact, or a combination of these. Therefore, in the second swelling study, swollen weights for both OPF formulations that had been dried and swollen (S1 in Figure 4.1) and those that had been swollen first, then dried, and then swollen again (S2' in Figure 4.1) were compared. There was no significant difference in swelling between these treatments, indicating continued crosslinking due to the presence of initiator during drying was not a primary cause of the observed differences in swelling.

To investigate the possibility of the formation of crystalline phases during the drying process, DSC analysis on several sample types was performed (Figure 4.3). After determination of the melting point of dry, linear OPF (10K: 60.4±0.8 °C; 1K: 39.4±0.9 °C), the DSC data from hydrated OPF 1K and 10K hydrogels that had either been vacuum-dried and swollen, or swollen immediately after crosslinking, was examined in this range. If crystallites had been formed within the macromer chain, endotherms in the melting point range would be apparent. However, the only endothermic peaks visible in these samples occurred near 100°C, which represents the evaporation of the water phase. From published experiments using similar methods, melting endotherms were observed via DSC with PEG-containing polymers in the dry state having a degree of crystallinity of 2% and higher [119, 120]. While it should be noted that, with the hydrated hydrogels,
the fraction of polymer present is lowered due to the large amount of water in the sample, based on the endotherms generated, there was no detectable evidence of crystallite formation in these specimens. Combined with the results of the second swelling study, these experiments suggest that the reduction in swelling was primarily due to network conformation changes resulting in increased chain entanglements upon drying.

The second swelling study also revealed that for all treatments, the sol fraction remained constant over multiple swelling-drying cycles (Figure 4.2 B), suggesting that most leachable (and potentially non-biocompatible) products were removed after one cycle. In addition, the results demonstrate that our laboratory is able to generate hydrogels with varied mesh sizes [116] that show relatively constant swelling characteristics after sol fraction loss. This indicates that OPF hydrogels can be synthesized with predictable material properties that may be customized for a variety of GTR applications.

Results from cell attachment assays at both 4 and 24 hrs established that there was significantly less cell adhesion to OPF hydrogels than to the TCPS control. The 1K VDES samples, which showed the highest attachment of the hydrogel samples at both time points, had attachment values approximately 50-60% of the control. While, in these experiments, human dermal fibroblasts were used as they have a similar morphology to cell types whose migration may be halted by a GTR membrane, similar results were found in previous studies with unmodified OPF and marrow stromal osteoblasts [113], suggesting that this phenomenon is not cell type-dependent. These findings suggest that, even after drying, OPF materials retain the ability to significantly reduce non-specific cell attachment, an important quality for use in GTR membranes.
Overall, the hydrogel samples that exhibited greater swelling demonstrated lower cell attachment. While this was also true for the 1K VD-ES specimens, the unique undulated surface morphology seen in the fluorescent microscopy images (domain size on order of hundreds of microns; Figure 4.5 F) may also be responsible for the significantly higher cell attachment for this sample group. Therefore, it is important to consider not only how the drying process affects the final bulk material properties, but also the surface properties of materials used for pre-formed scaffolds.

When considering cell attachment over time, there was either no difference (1K ES) or an increase in number of cells on the surface at the later time point, except in the case of the 10K ES samples. This could be explained by initial attachment of fibroblasts to the 10K ES samples, and subsequent detachment as the cells could not form strong interactions with the hydrogel surface. This is supported by the images of cells stained with LIVE/DEAD reagent (Figure 4.5 A-D), which demonstrate that all of the cells attached to the hydrogels were alive, but appeared rounded, rather than having an elongated morphology typical for fibroblasts. These images indicate that the OPF hydrogel surfaces have the ability to reduce non-specific cellular adhesion through influencing cellular morphology.

CONCLUSIONS

Results from this study demonstrate that drying history has a significant impact on swelling properties of OPF hydrogels. The decrease in swelling evident when hydrogels were dried before swelling is due primarily to increased chain entanglements rather than polymer crystallite formation or further crosslinking in the dried state. In addition to swelling properties, drying history also influences non-specific cell adhesion to the
hydrogels. The significantly higher cell attachment to OPF hydrogels that were dried before swelling correlates to reduced final swelling and, in some cases, altered surface morphology. By changing the PEG molecular weight used in synthesis, OPF hydrogels may be tailored to provide desired material properties and reduce non-specific cell adhesion for either injectable or pre-formed applications, thus providing a potential alternative GTR material for a variety of clinical procedures.
**Table 4.1**: Results comparing effects of drying methods on OPF hydrogel swelling properties. Results are presented as means ± standard deviation, n=4-5.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Swelling Ratio</th>
<th>Sol Fraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swollen First**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10K ES-AD</td>
<td>13.81±0.08</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td>1K ES-AD</td>
<td>7.88±0.34</td>
<td>0.38±0.01</td>
</tr>
<tr>
<td>10K ES-VD</td>
<td>13.94±0.35</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>1K ES-VD</td>
<td>8.99±0.47</td>
<td>0.39±0.03</td>
</tr>
<tr>
<td>Dried First</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10K AD-ES</td>
<td>6.91±0.21</td>
<td>0.50±0.02</td>
</tr>
<tr>
<td>1K AD-ES</td>
<td>3.42±0.05</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>10K VD-ES</td>
<td>6.53±0.12</td>
<td>0.37±0.01</td>
</tr>
<tr>
<td>1K VD-ES</td>
<td>2.26±0.08</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

*The average dried weight from the corresponding dried-first samples was used to calculate sol fraction for swollen-first samples.

**All swollen-first samples showed significantly higher swelling than their dried-first counterparts, regardless of OPF type or drying method. In addition, for each treatment, OPF 10K exhibited significantly higher swelling than OPF 1K (p≤ 0.05 for all statistical tests).
**Figure 4.1:** Methods for swelling studies. OPF hydrogel discs were either dried overnight at <1 torr and then swollen to equilibrium swelling (VD-ES), dried overnight at 760 torr, then dried for an additional 12 hours at <1 torr and then swollen (AD-ES), or swollen first and then dried at either <1 torr or 760 torr (ES-VD or ES-AD). The first swelling study was completed only through Cycle 1, while the second study involved all 3-4 cycles. In the repeated swelling/drying study, the fold swelling was calculated for each cycle i (using weights of S1 or S1' and D1 or D1'), while the sol fraction was determined with reference to the original dry weight before swelling (D0). Comparison of weights from S1 and S2' samples were carried out to elucidate causes of swelling differences observed between the treatments.
\textbf{Figure 4.2}: Fold swelling (A) and sol fraction (B) of OPF hydrogels with various drying histories over 3-4 cycles of repeated drying and swelling (± standard deviation, n=4-5). Sol fraction was calculated with reference to the sample’s original dry weight before swelling.
**Figure 4.3:** Representative DSC graphs of hydrated OPF 10K (A) and OPF 1K (B) hydrogels that had been dried before swelling and the corresponding linear OPF molecules (n=3 per sample type).
**Figure 4.4:** Percent of seeded human dermal fibroblasts attached to tissue culture polystyrene (TCPS, positive control) and OPF hydrogels after 4 and 24 hours (± standard deviation, n=4). * indicates that there was significantly more cell attachment to TCPS than any of the hydrogel formulations at both time points. Of the OPF samples, the 1K dried-first gels (**) exhibited significantly more cell adhesion than the other formulations at both time points, and, at 24 hrs, the 10K dried-first samples (#) showed significantly higher attachment than the hydrogels that were swollen first (p ≤ 0.05).
Figure 4.5: Fluorescent microscope images of OPF surfaces after 24 hours of cell attachment (scale bar 200 μm). The green background is due to autofluorescence of OPF. A-D) Fibroblasts stained with LIVE/DEAD reagent appear live, but rounded (100X). A) 10K OPF that has been swollen prior to introduction of cells. B) Corresponding 1K OPF. C) 10K OPF that has been vacuum-dried and then swollen prior to introduction of cells. D) Corresponding 1K OPF. E-F) Surfaces of 10K VD-ES and 1K VD-ES hydrogels, respectively (40X). Undulations are seen only on the OPF 1K dried-first samples (domain size on order of hundred of microns). All 10K samples and the 1K specimens that were swollen directly after fabrication have similar surface characteristics to E).
CHAPTER V

IN VITRO CYTOTOXICITY OF REDOX RADICAL INITIATORS FOR CROSSLINKING OF OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) MACROMERS

ABSTRACT

A novel hydrogel system based on oligo(poly(ethylene glycol) fumarate) (OPF) is currently being investigated as an injectable carrier for marrow stromal cells (MSCs) for orthopaedic tissue engineering applications. This hydrogel is crosslinked using the redox radical initiators ammonium persulfate (APS) and ascorbic acid (AA). In this study, two different persulfate oxidizing agents (APS and sodium persulfate (NaPS)) with three reducing agents derived from ascorbic acid (AA, sodium ascorbate (Asc) and magnesium ascorbate-2-phosphate (Asc-2)) and their combinations were examined to determine the relationship between pH, exposure time, and cytotoxicity for rat MSCs. In addition, gelation times for specific combinations were determined using rheometry. pH and cell viability data after 2 hrs for combinations ranging from 10 to 500 mM in each reagent showed that there was a smaller pH change and a corresponding higher viability at lower concentrations, regardless of the reagents used. At 10 mM, there was less than a 1.5 unit drop in pH and greater than 90% viability for all initiator combinations examined. However, MSC viability was significantly reduced with concentrations of 100 mM and higher of the initiator combinations. At 100 mM, exposure to NaPS/Asc-2 resulted in significantly more live cells than APS/AA or NaPS/Asc, but, at this

*This chapter has been submitted for publication as: J.S. Temenoff**, H. Shin**, D.E. Conway, P.S. Engel, A.G. Mikos, "In vitro cytotoxicity of redox radical initiators for crosslinking of oligo(poly(ethylene glycol) fumarate) macromers," Biomacromolecules. **Equal contributions to this work.
concentration, NaPS/Asc-2 exhibited significantly longer OPF gelation onset times than APS/AA. At all combination concentrations, exposure time (10 min vs. 2 hrs) did not significantly affect MSC viability. These data indicate that final pH and/or radical formation have a large impact on MSC viability and that multiple, intertwined testing procedures are required for identification of appropriate initiators for cell encapsulation applications.

INTRODUCTION

In recent years, a variety of hydrogel materials have been explored for use as cell carriers in orthopaedic tissue engineering. Such carrier materials play an important role in promotion of tissue regeneration through localized retention of cells at the defect site. Both natural- and synthetic-based materials have been studied as a means of cell transplantation to improve healing of cartilage and non-load bearing bone defects [57-60, 63, 64, 74, 121-130]. Natural materials previously employed to encapsulate cells include collagen [57, 121, 122], fibrin [58, 59, 123, 124], alginate [60] and derivatives [125], chitosan [126], and agarose [127]. Additionally, synthetic materials based on acrylamides [128], poly(vinyl alcohol) (PVA) [129], poly(ethylene oxide) (PEG) [63, 64, 74] and copolymers of PEG and poly(propylene oxide) [130] have been studied for this application. These synthetic hydrogels possess key advantages such as ease of mass production, assurance of pathogen removal and tailorability for specific applications [71]. In addition, PEG and PVA-based hydrogels have been photopolymerized using ultraviolet light to form a three-dimensional structure around cells within seconds to minutes, allowing control of cellular distribution within the gel [74, 129].
Our laboratory is currently developing a novel, biodegradable, \textit{in-situ} crosslinkable, synthetic hydrogel material based on oligo(poly(ethylene glycol) fumarate) (OPF) for use as an injectable carrier for marrow stromal cells (MSCs) to aid regeneration of orthopaedic injuries. This oligomer can be crosslinked in the presence of the water-soluble redox initiators ammonium persulfate (APS) and ascorbic acid (AA) to form a solid network [116]. Since initiation is a bimolecular reaction, gelation is dependent on temperature and concentration, rather than the presence of light, to commence curing. While this may be advantageous for certain clinical applications where light penetration is limited, the cytocompatibility of this initiator system must be evaluated before it can be used as part of a cell transplantation paradigm.

Previous studies in our laboratory have demonstrated the cytocompatibility of the linear OPF molecule [131] and the biocompatibility of the resulting crosslinked hydrogel [131, 132], but, because MSCs are present during the crosslinking reaction, the cytocompatibility of all components that may interact with the cells must be determined. Therefore, this study was undertaken to evaluate the cytocompatibility of water-soluble initiators employed to crosslink OPF.

In particular, the pH of these initiators was singled out as a concern for potential cytotoxic effects. Thus, in these experiments, the reducing agent AA, its salt, sodium ascorbate (Asc), or a salt found to be more stable in cell culture conditions, magnesium ascorbate-2-phosphate (Asc-2, see Figure 5.1) was combined with the oxidizing agent APS or sodium persulfate (NaPS). Various combinations at concentrations from 1 mM to 1000 mM were examined to determine the relationship between pH, exposure time, and cytotoxicity for rat MSCs. Specifically, the effect of initiator concentration on pH in
media over 2 hrs in an incubator, how these changes in pH affected cell viability, and how exposure time (10 min vs. 2 hrs) to each of these solutions impacted viability were studied. Additionally, the time to onset and completion of gelation for various initiator combinations, important for seeding homogeneity in future cell encapsulation procedures, was assessed via rheometry.

**MATERIALS AND METHODS**

**pH Studies**

Solutions of the oxidizing agents NaPS (Sigma-Aldrich, St. Louis, MO) and APS (EM Science, Gibbstown, NJ), the reducing agents, AA, Asc, and Asc-2 (all from Sigma-Aldrich), as well as combinations of APS/AA, NaPS/Asc, and NaPS/Asc-2, were made in primary media (DMEM high glucose (Gibco, Grand Island, NY)) supplemented with 10% v/v fetal bovine serum (Gemini, Calabasas, CA) and 1% v/v antibiotics containing penicillin and streptomycin (Gibco). The individual initiators were prepared in triplicate in concentrations from 1-1000 mM, while the combinations were made from 1-500 mM. Due to limited solubility, all solutions containing Asc-2 were prepared only from 1-100 mM. The initial pH and pH after 2 hrs was recorded using an electronic pH meter (AP5, Fisher Scientific, PA) for the individual initiator components. The kinetics of pH change for the combination solutions was monitored at 0, 10, 20, 30, 60, 120 and 240 min as the samples were maintained in an incubator (37°C, 95% relative humidity, 5% CO₂). Media (no initiators added) placed concurrently in an incubator served as a control. The pH change for each solution was calculated using the following equation:

\[
\Delta \text{pH} = \text{pH (each sample solution)} - \text{Avg(\text{pH (control)})},
\]

where \( \text{Avg(\_)} \) represents the average value of the parameter in parentheses.
Viability Studies

Marrow Stromal Cell (MSC) Culture

MSCs were isolated from 6-week-old male Wistar rats as previously described [44]. Briefly, femurs and tibias were excised from the rats under aseptic conditions and the marrow was flushed from the bone with primary media. After centrifugation for 8 min at 1250 rpm, the cell pellet was re-suspended and seeded in T-75 culture flasks. Cell cultures were maintained in the incubator for 6 days prior to use in the study. Non-adherent cells were removed and the media was changed after 3 days in culture.

Determination of Viability Fraction

The amount of viable cells after exposure to initiator components was quantified as described previously [131]. Briefly, after trypsinization (0.05% Trypsin/0.53 mM EDTA, Gibco) MSCs were plated in 96-well plates at 40,000 cells/cm² and allowed to attach for 24 hrs. At this time, the media was removed and replaced with filter-sterilized (0.2 μm filter, Nalge, Rochester, NY) initiator solutions in primary media (n = 3 wells/treatment).

In order to study how MSCs responded to length of exposure to initiator components, two experiments were performed. In the first, cells were exposed to the initiators singly or combinations of APS/AA, NaPS/Asc, and NaPS/Asc-2 (final concentrations in the media were 500 mM, 100 mM, and 10 mM for each component) continuously for 2 hrs. In the second experiment, the initiator-containing media (final concentrations 100 mM and 10 mM for each component) was removed after 10 min, the wells were rinsed once with primary media, fresh primary media was added, and the plate was incubated for an additional approximately 1 hr 50 min. For both experiments, 2 hrs
after the introduction of the original experimental media, the media in each well was aspirated and the LIVE/DEAD reagent (4 μM ethidium homodimer-1 (EthD-1) and 2 μM calcein AM, Molecular Probes, Eugene, OR) was added. After a 30 min incubation at room temperature, the fraction of live and dead cells was quantified by recording fluorescence via a plate reader (FLx800, BIO-TEK Instruments, Winooski, VT) equipped with 485/528 (Excitation/Emission) filter sets for calcein AM (live cells) and 528/620 (Excitation/Emission) filters for EthD-1 (dead cells).

The wells in which MSCs were cultured without exposure to experimental media served as the positive (live) control. For the negative (dead) control, MSCs were exposed to 70% (v/v) methanol solution for 30 min prior to addition of the LIVE/DEAD reagent. After measurement, the fluorescence of each sample well was normalized by that obtained from the positive and negative controls to obtain the fractions of live and dead cells according to the equations below:

\[
\text{Fraction of live cells: } \frac{F_{ls}}{F_{lc}}
\]

Where \( F_{ls} \) is: \( \text{fluorescence}_{(\text{sample})} - \text{Avg}(\text{background fluorescence}_{(\text{dye, no cells})}) \).

\( F_{lc} \) is: \( \text{Avg}(\text{fluorescence}_{(\text{live control})}) - \text{Avg}(\text{background fluorescence}_{(\text{dye, no cells})}) \),

where \( \text{Avg}(\ ) \) represents the average value of the parameter in parentheses.

Fraction of dead cells: \( \frac{F_{ds}}{F_{dc}} \)

Where \( F_{ds} \) is: \( \text{fluorescence}_{(\text{sample})} - \text{Avg}(\text{background fluorescence}_{(\text{dye, no cells})}) \).

\( F_{dc} \) is: \( \text{Avg}(\text{fluorescence}_{(\text{dead control})}) - \text{Avg}(\text{background fluorescence}_{(\text{dye, no cells})}) \).
After quantification, representative wells were imaged via a fluorescence microscope (Axiovert 135 with FITC and Texas Red filter sets, Zeiss, Thornwood, NY) equipped with a 35-mm camera (Contax 167 MT, Kyocera Electrical Inc., Somerset, NJ).

**Rheometry Studies**

**OPF Synthesis and Characterization**

Two formulations of OPF were synthesized from PEG of different initial number average molecular weight: ~8,480 g/mol (designated OPF 10K) or ~870 g/mol (OPF 1K), following established procedures [77]. Briefly, PEG (Sigma-Aldrich), was distilled and dissolved in dichloromethane (distilled before use) (Fisher Scientific, Pittsburgh, PA). Fumaryl chloride (distilled before use, Acros, Pittsburgh, PA) and triethylamine (Sigma-Aldrich) were then added drop-wise to the PEG solution, held at approximately 0°C. The reactions were allowed to stir for 48 hrs at room temperature under nitrogen. The excess solvent was evaporated, and the OPF was recrystallized twice from ethyl acetate (Fisher Scientific). After precipitation with ethyl ether (Fisher Scientific), the resulting powder was vacuum dried at <5 torr and stored in a sealed container at -20°C until use.

After synthesis, the OPF was characterized via gel permeation chromatography (GPC). A GPC system (Waters Model 410, Milford, PA) with a differential refractometer was used to determine molecular weight of both the PEG starting material and the resulting OPF. After dissolution in chloroform, molecular weights were determined from elution time (Waters column, 50-100,000 Da range) based on a calibration curve generated with monodisperse PEG standards. Three samples of each type were evaluated.


**Determination of Gelation Time**

After synthesis, OPF 10K or OPF 1K was dissolved in phosphate-buffered saline (PBS, Gibco) and mixed with the crosslinker poly(ethylene glycol) diacrylate (PEG-DA, \( M_n \), 575, Aldrich) (25 wt% initial polymer, 2 OPF: 1 PEG-DA by weight) [116]. Subsequently, the redox initiator combination APS/AA or NaPS/Asc-2 (final concentrations of each initiator component were 100 mM or 10 mM) was introduced. The complex viscosity (\( \eta^* \)) of these solutions over time was calculated from a dynamic oscillatory test using a rheometer (Rheolyst AR1000, TA Instruments, New Castle, DE) with an 8 cm flat plate held at 37°C. Data points were recorded approximately every 30 s and PBS was added every 10 min to maintain hydration of the crosslinking hydrogel over the course of the experiment. Samples were run until \( \eta^* \) plateaued or for 2 hrs if no plateau was seen. Gelation onset was regarded as the first time at which there were at least 4 consecutive points with \( \geq 20\% \) change in complex viscosity (compared to the previous point). Gelation completion was determined to be the first time at which there were at least 4 consecutive points with less than 1% change in \( \eta^* \). Samples were run in triplicate.

**Statistical Analysis**

Data from all studies were analyzed using ANOVA and Tukey’s Multiple Comparison Test or an unpaired Student’s t-Test (\( p \leq 0.05 \)). The results are reported as mean ± standard deviation.

**RESULTS**

**pH Studies**
pH measurements were completed on individual initiator components in concentrations from 1-1000 mM and for redox pairs from 1-500 mM. However, for ease of comparison with viability and rheometry data, only results from 10 mM, 100 mM, and 500 mM are presented in Table 5.1 and Figure 5.2 and summarized in this section. Immediately after preparing each solution (0 min), both oxidizing agents had significantly lower pH at higher concentrations, although there was no difference in pH between 100 mM and 500 mM NaPS (Table 5.1). AA showed a significantly greater pH change with increasing concentration, while the other reducing agents exhibited no difference in pH with varying concentrations.

Comparing between individual components at the same concentration, APS demonstrated a significantly lower pH than NaPS, regardless of concentration. Similarly, AA consistently showed a significantly greater pH drop than the other two reducing agents. When examining the individual initiator components and their resulting combinations (combination data not shown), it was found that at 500 mM, there was less pH change with AA than APS, which, in turn demonstrated a significantly greater pH drop than the APS/AA. While both NaPS and Asc had pH near 7, their combination had a significant pH drop at 500 mM (the NaPS/Asc-2 combination was not possible at this concentration). At 100 mM of each reagent, all changes in pH were statistically equivalent for the APS/AA combination and constituents, while the NaPS/Asc followed the same trends seen at 500 mM. Both components and the combination of NaPS/Asc-2 resulted in a pH near 7 at 100 mM. At 10 mM, all combinations and constituent reagents demonstrated a pH within one unit of 7.
After 10 min in an incubator, the pH of the combined initiator solutions were measured (see Figure 5.2 B). All combinations tested showed a significantly greater pH change with increasing concentration. At all concentrations, APS/AA had a significantly larger pH change than either of the combinations with NaPS. At both 100 mM and 10 mM, NaPS/Asc demonstrated a significantly greater pH drop than NaPS/Asc-2.

At 2 hrs, the pH of the individual components and the initiator combinations were again tested (Table 5.1). Statistical trends were identical to those seen at 0/10 min, except in a small number of cases. For individual initiator components, both APS and NaPS showed significantly lower pH at higher concentrations. When examining the individual components and their resulting combinations, at 500 mM, there was a significantly larger pH change with APS than with AA, but both demonstrated a significantly smaller pH drop than APS/AA. At 100 mM each reagent, the pH change for APS and AA were statistically equivalent and were both significantly less than that for their combination. At 10 mM, all combinations and constituent reagents demonstrated pH within 1.5 units of 7.

The pH of the initiator components was also studied as a function of time (Table 5.1). At the 500 mM concentration, APS demonstrated a significantly larger pH change at 0 min than 2 hrs, while NaPS remained within one unit of pH 7. AA also demonstrated a significantly greater pH change at 0 min than 2 hrs, while Asc remained within one unit of pH 7 throughout the experiment. At 100 mM, APS demonstrated statistically equivalent pH drops at both time points. AA showed a significantly greater pH change at 2 hrs than 0 min. The other reducing agents, as well as NaPS, had a pH within one unit of 7 throughout the experiment.
In addition, the pH of the initiator combinations was examined over time (Table 5.1). At 500 mM, both the APS/AA and NaPS/Asc exhibited a larger pH change at 2 hrs than 0 or 10 min, which were statistically equivalent. At 100 mM, all combinations showed a greater pH change over time.

**Viability Studies**

In these studies, both the fraction of live and dead cells were calculated with reference to appropriate controls. The fraction of dead cells was used only to confirm the trends seen in the data from the live assay [131], and thus is not presented here. The fraction of viable cells after various treatments is shown in Figure 5.2, with corresponding fluorescence microscopy images displayed in Figure 5.3.

For the individual initiator components, after 2 hrs of continuous exposure (Figure 5.2 A), NaPS demonstrated decreasing viability with increasing concentration, while at 100 mM and higher, a precipitate was seen coating the wells of the APS-treated samples, preventing accurate fluorescence detection. Among the reducing agents, significantly less viability was seen with increasing concentrations of AA and Asc. There was no statistical difference in viability between 100 mM and 10 mM Asc-2, which both had viability fractions near 1.

Comparing individual components at the same concentration, NaPS and APS could only be examined at 10 mM due to coating of the APS-treated wells. At this concentration, there was no statistical difference in viability between the oxidizing agents. AA exhibited significantly lower viability than the other two reducing agents at both 500 and 100 mM. At 100 and 10 mM, Asc-2 had a significantly higher fraction live cells than the other two reducing agents.
In examining the wells exposed to initiator combinations, a coating was also found on the bottom of samples with APS/AA or NaPS/Asc at concentrations higher than 10 mM. The NaPS/Asc-2 combination exhibited significantly higher viability with decreasing concentration and, at 10 mM, all wells demonstrated viability of approximately 1.

When comparing the individual initiator components and their resulting combinations, at 100 mM, NaPS demonstrated significantly fewer live cells than Asc-2, and either reagent alone showed higher viability than their combination (other treatments could not be compared due to surface coating). At 10 mM, AA exhibited significantly less viability than APS or the combination, which were statistically equivalent. Similarly, Asc showed significantly fewer viable cells than NaPS or the combination, both of which had viability of approximately 1. With NaPS/Asc-2 at this concentration, both reagents and their combination resulted in viability of 0.98 or higher.

In a second study, MSCs were exposed to the various reagents only for 10 min, and viability was assessed after 2 hrs (Figure 5.2 B). In these experiments, viability was tested only at 100 mM and 10 mM concentrations of each reagent. For the individual initiator components, there was no statistical difference in fraction of live cells between concentrations for either oxidizing agent, and all of these values fell near 1. There was significantly less viability at the higher concentration of AA, while there was no statistical difference in viability for Asc or Asc-2, both of which demonstrated viability fractions near 1 regardless of concentration.

In this study, no coating appeared on wells containing initiator combinations, as was observed after 2 hrs of continuous exposure. Both APS/AA and NaPS/Asc exhibited
greater viability at the lower concentration. There was no statistical difference with NaPS/Asc-2, which maintained a viability fraction near 1 at both initiator concentrations. Comparing combinations at a given concentration, NaPS/Asc-2 demonstrated significantly higher viability than the others at 100 mM, but at 10 mM, all combinations produced live cell fractions near 1.

When examining the individual initiator components and their resulting combinations, at 100 mM, APS demonstrated significantly more live cells than AA, and either reagent alone showed significantly higher viability than their combination. While the viability for NaPS and Asc was determined statistically equivalent, both exhibited a significantly greater number of viable cells than their combination. The NaPS/Asc-2 combination (100 mM) and all combinations at 10 mM showed results similar to data from 2 hrs of continuous exposure.

The results of the 10-minute and 2-hour exposure studies were compared to decipher the effects of exposure time on viability. At 100 mM, it was found that there was significantly greater viability after 10 min than 2 hrs of exposure for all oxidizing agents and all reducing agents except Asc-2. In this case, the viability ratio remained approximately 1, regardless of time in culture. At 10 mM, significantly greater viability was seen at 10 min than 2 hrs for AA and Asc, while Asc-2 and the oxidizing agents demonstrated viability near 1 for both exposure times. In examining the initiator combinations at 10 mM (due to well coating for some samples at 100 mM), all exhibited viability fractions near 1 at both 10 min and 2 hrs continuous exposure.
Rheometry Studies

OPF Synthesis and Characterization

GPC analysis revealed that the OPF 10K synthesized had an average \( M_n \) of 11,600 \( \pm \) 190, while the corresponding PEG used for macromer synthesis had an average \( M_n \) of 8,480 \( \pm \) 53. The average \( M_n \) of OPF 1K was 2,860 \( \pm \) 40 and the PEG used to produce this macromer had an average \( M_n \) of 870 \( \pm \) 20.

Determination of Gelation Time

Gelation time was determined for OPF 10K and 1K with the initiator combinations APS/AA and NaPS/Asc-2 at 100 mM and 10 mM and the results are summarized in Figure 5.4. Comparing gelation onset times for OPF 10K, it was found that there was no significant difference between the two concentrations of APS/AA, but that the higher NaPS/Asc-2 concentration did result in a significantly earlier onset than the lower concentration of this combination. The onset of both NaPS/Asc-2 concentrations was significantly later than either of the APS/AA combinations. At 10 mM, NaPS/Asc-2 did not show evidence of gelation onset over 2 hrs. Gelation completion time was significantly shorter with 100 mM than 10 mM APS/AA, and both APS/AA concentrations demonstrated earlier completion times than either of the NaPS/Asc-2 combinations, which did not finish crosslinking within 2 hrs. The same statistical trends were seen with the OPF 1K samples, except that there was no difference in gelation completion time between the two concentrations of APS/AA.

Examining the onset times between OPF types at 100 mM, APS/AA demonstrated no significant difference for OPF 10K vs. OPF 1K. However, it was found that there was a significantly later onset with NaPS/Asc-2 for OPF 1K than OPF 10K. At the 10
mM initiator concentration, there was no statistical difference between OPF 10K and OPF 1K for APS/AA (NaPS/Asc-2 was not compared as there was no gelation onset). Unlike the onset, gelation completion values were significantly later for OPF 1K than OPF 10K with 100 mM APS/AA (NaPS/Asc-2 was not compared as there was no completion of gelation). At 10 mM, the trends for completion were identical to those seen for gelation onset.

**DISCUSSION**

In these studies, AA and two of its salts, Asc and Asc-2 (see Figure 5.1), were combined with the oxidizing agents APS or NaPS to examine the relationship between pH, exposure time, and cytotoxicity for rat MSCs. In particular, the individual components and the combinations APS/AA, NaPS/Asc and NaPS/Asc-2 were tested at concentrations from 10-500 mM in cell culture media for change in pH over 2 hrs in an incubator. Subsequently, the effect of pH change and exposure time on cell viability, and the effect of initiator combination and concentration gelation time were studied. For applications using OPF as an injectable cell carrier, MSCs would be exposed to all system components during crosslinking, and therefore, it is essential that all agents present, including initiators, display minimal cytotoxic effects.

The APS/AA initiator pair possesses the advantages of water-solubility and the need for only a modest increase in temperature to produce radicals that will begin the crosslinking reaction [133-136]. However, a possible difficulty may be the low pH of both the APS and AA. Therefore, similar molecules more likely to have pH near neutral were chosen for this study in order to elucidate the effects of pH on cell viability. For comparison with APS, NaPS was selected because it retains the strongly-oxidizing
persulfate ion, essential to the initiating reaction [135, 136]. For AA, its salt, Asc was chosen. However, since both AA and Asc are known to be quickly oxidized to a metabolically inactive form in aqueous conditions at 37°C [137-141], a more stable derivative of the salt, Asc-2, containing a phosphate group added to the carbon in the second position [137-143] (see Figure 5.1) was also examined. It should be noted that the reaction that occurs to initiate crosslinking with the persulfate-ascorbate system in aqueous media may not be a straightforward redox reaction [135, 136]. However, in the present discussion, AA and its derivatives are termed "reducing agents" and APS and NaPS are designated "oxidizing agents."

The concentration range examined in these experiments was based on previous work that demonstrated the formation of stable hydrogels with several types of OPF using 100 mM APS and AA [116]. Therefore, this concentration, as well as those both higher (500 mM) and lower (10 mM) were chosen for this study. Preliminary work with these initiators (see Figure 5.4) also indicated that gelation would commence within 2 hrs for all combinations used in these experiments (100 mM). Thus, 2 hrs was the longest exposure time examined in the viability assessment. However, in an ideal injectable system, gelation would begin immediately in order to maintain the spatial orientation of the encapsulated cells [74]. Therefore, a 10 min exposure time was also examined in viability studies to include the shortest gelation time expected.

\textit{pH Studies}

pH studies confirmed the acidity of APS and AA, while the other reagents individually remained near pH 7 (Table 5.1). After 2 hrs in the incubator, APS, NaPS and AA demonstrated lower pH with increasing concentration in the media. Examining
the combinations over time, both APS/AA and NaPS/Asc produced a large pH drop immediately after preparation, while NaPS/Asc-2 had a smaller initial change, but continued to decrease over a 2-hr period. These data support the formation of an ascorbate radical anion, which is known to be strongly acidic [144]. Formation of such a species would be consistent with the fact that NaPS and Asc remained neutral over 2 hrs separately, but, when combined, resulted in a significant pH drop. At 2 hrs, an overall drop in pH was observed with NaPS/Asc-2 at 100 mM, although it was significantly less than for the other two combinations (APS/AA: -6.63+/-0.08, NaPS/Asc: -6.07+/-0.01, NaPS/Asc-2: -2.37+/-0.03). This suggests that the radical anion is slowly generated even with this combination, which would be possible if the phosphate group on the ascorbate molecule were hydrolyzed during the reaction. Although this ascorbate derivative is known to be stable for days in aqueous conditions [139], nothing is known about the stability of the phosphate functionality in the presence of oxidizing agents such as persulfates. At 10 mM, all the reagents and combinations exhibited a fairly constant pH over time, and all remained within 1.5 units of neutral, even after 2 hrs in the incubator.

**Viability Studies**

Experiments involving 2 hrs of continuous exposure to the initiator reagents demonstrated that there was a smaller change in pH and a corresponding higher viability at lower concentrations of APS, AA, or the combinations APS/AA, NaPS/Asc and NaPS/Asc-2 (Figure 5.2 A). These results indicate that low pH decreases cell viability. However, in experiments involving initiator combinations, it was not possible to decouple the effects of pH and radical formation, as decreasing concentrations of initiators are likely to produce fewer radicals [135, 136]. In addition, APS or NaPS alone
may cause cell death due to their strongly oxidizing nature [145]. This is shown in the low viability (less than 1%) for NaPS 500 mM at 2 hrs, even though the pH of this solution remained approximately 7 throughout the experiment. AA and Asc, while often included in cell culture media to promote cell proliferation and collagen formation [138, 140, 141, 146], are also known to possess cytotoxic properties at concentrations in the mM range after hours to days in culture [139, 146, 147]. This has been attributed, in part, to the autooxidative properties of these molecules, which can result in the formation of radicals without the presence of persulfates [139, 141]. As in the case of NaPS, Asc, which remained near neutral pH over 2 hrs, showed a maximum viability of ~40% after this period, suggesting that factors other than pH play a role in cell death.

As revealed by the images from wells exposed to AA and its derivatives at 100 mM (see Figure 5.3), only the Asc-2-exposed cells maintained morphology similar to the positive control (Figure 5.3 F). In some cases with this reagent, the cells appeared more spread and fluoresced brighter than the positive control, which may contribute to viability fractions greater than 1 for these samples. In contrast, the AA wells showed rounded cells with little cytoplasm, often found in gathered in clumps (Figure 5.3 D), and, in the Asc wells, the cells maintained a more even distribution, but were not spread and lacked cytoplasmic space (Figure 5.3 E). Such morphology is often found in apoptotic cell death, and has been seen previously when human promyeloic leukemia cells were exposed to AA and Asc for up to 6 hrs, but was not found when these cells came in contact with Asc-2 containing media [139]. In this study, the decrease in viability was correlated with increased radical intensity from the decomposition of the AA or Asc molecules over time.
in culture [139]. Thus, a similar mechanism may explain the low viability fractions seen in these experiments in wells containing AA or Asc alone.

When combining the oxidizing and reducing agents, viability increased with decreasing initiator concentrations and only NaPS/Asc-2 demonstrated any significant viability at 100 mM after 2 hrs of continuous exposure. However, from the images of this combination (Figure 5.3 H), it is evident that the cells, although green (live), are contracted and do not cover the bottom of the well evenly. In contrast, the morphology of cells exposed to any of the combinations at 10 mM (not shown) appeared similar to that of the positive control.

Although in most cases, the fraction of dead cells confirmed the viability data presented here, it was found with certain samples, the sum of live and dead fractions was less than one. Specimens in which the total was below 0.9 included APS 500mM, APS 100mM, NaPS 500 mM, Asc 500 mM, and all combinations at 100 mM or higher concentrations. This can be explained in part by coating of the well plates seen with APS at 100 mM and higher, and combinations of APS/AA or NaPS/Asc at 100 mM and greater concentrations. Such coating is most evident in the image of APS/AA-treated cells at 100 mM (Figure 5.3 G) and may be a precipitate formed due to the interaction of the reagents with components of the media. Additionally, in many samples, the MSCs appeared small and rounded, and in some cases, there were large blank areas where cells may have been detached during the rinsing/staining steps (depicted for wells with APS 100 mM, AA 100 mM, and Asc 100 mM (Figure 5.3 C-E)). This could also contribute to low total fluorescence for certain treatments.
In the second viability study, MSCs were exposed to each reagent for 10 min, and viability was assessed at 2 hrs. Due to the high cytotoxicity of the 500 mM concentration of all solutions in the first study, the second study was conducted at 100 mM and 10 mM only. In addition, long-term cell viability was not tested in these studies, as these experiments represent an initial screening process to identify potentially cytotoxic initiator combinations.

Shorter exposure time did result in significantly higher viability for the 100 mM concentrations of all the individual components except Asc-2, which demonstrated high viability at both time points. However, when combined, viability results were similar between 10 min and 2 hrs (Figure 5.2). As in the first study, cell death may be attributed to a combination of pH change and radical formation from the combinations. Cellular morphology (pictures not shown) was similar to that seen at 2 hrs, but with less pronounced cell shrinkage and fewer non-viable cells in many cases. Unlike the previous study, no well coating was observed after 10 min continuous initiator exposure. In addition, the only sample where the sum of live and dead fractions was less than 0.9 was APS/AA 100 mM. With the shorter exposure time, this could be due to changes in morphology and/or detachment of dead cells during rinsing, rather than plate coating.

**Rheometry Studies**

The molecular weight of the OPF macromers was determined by GPC. As previously reported, OPF hydrogels with varied material properties can be created through changing the PEG molecular weight used in the synthesis of the oligomer [77, 116]. Therefore, for rheometry studies, OPF of PEG chains with initial molecular weights of approximately 1K and 10K were selected to determine the effects of initiator
combination, initiator concentration, and OPF type on crosslinking time. In these experiments, only the combinations APS/AA and NaPS/Asc-2 were examined, because, from the viability studies at 100 mM, they represent non-cytocompatible (APS/AA) and potentially cytocompatible (NaPS/Asc-2) initiator systems. Crosslinking was monitored over 2 hrs to facilitate comparison with the cell viability and pH data.

For the APS/AA system, gelation onset occurred within ~5 min at 37°C regardless of concentration or OPF type (Figure 5.4). However, gelation was completed in approximately 35 min for OPF 10K, while up to 50 min was required for OPF 1K, indicating that OPF molecular weight does impact gelation properties of these hydrogels. Observations of the gels suggest that a solid is formed before the plateau value is reached, thus potentially holding the encapsulated cells in the desired location, so it may not be necessary to maintain the gels undisturbed for the entire gelation time. However, the final mechanical properties of these gels may be affected by total crosslinking time [148].

In contrast to the APS/AA system, the NaPS/Asc-2 only demonstrated onset of gelation during the 2 hrs tested at 100 mM, and showed no evidence of gelation completion, irrespective of initiator concentration or OPF type. In these cases, the gels formed were soft and difficult to manage, suggesting that this is not an effective initiator system for OPF hydrogels at 37°C on this time scale. Such results emphasize the need for a series of experiments relating exposure time, viability and crosslinking properties when developing new injectable systems for cell encapsulation. Optimization of these properties to reduce cell death while maintaining a clinically-relevant crosslinking time is essential to the success of such materials. From these studies, the initiator system that best meets all required criteria is 10 mM APS/AA. This initiator demonstrates minimal
cytotoxicity, even after 2 hrs of continuous exposure to MSCs, and provides complete crosslinking within 1 hr for both OPF types tested. However, in the rheometry experiments, PEG-DA with nominal molecular weight of 575 was used as a crosslinker. While this PEG-DA molecular weight was chosen in order to maintain consistency with previous work involving mechanical testing of OPF hydrogels [116], it was found in a concurrent cytotoxicity study to be non-cytocompatible [131], and thus is unsuitable for use in cell encapsulation applications. Therefore, further experiments are needed to confirm that similar crosslinking times could be obtained with a higher molecular-weight PEG-DA, shown in other experiments to be cytocompatible [131].

CONCLUSIONS

Results from these experiments indicate that pH drop and MSC viability after exposure to combinations of redox radical initiators were concentration-dependent. At 10 mM, there was less than a 1.5 unit change in pH and greater than 90% viability for all initiator combinations examined. However, pH drop and/or radical formation caused significant cell death after exposure to concentrations of 100 mM and higher APS/AA and NaPS/Asc. At all combination concentrations, exposure time (10 min vs. 2 hrs) did not have a significant effect on viability. The NaPS/Asc-2 initiation system, which was more cytocompatible than the others, did not form fully crosslinked gels within 2 hrs at 37°C, as assessed by rheometry. These data highlight the need for multiple, intertwined testing procedures for identification of initiators that would be appropriate for use in cell encapsulation. This study, in conjunction with other cytotoxicity experiments completed for this system, suggest that, after optimization of crosslinking parameters, OPF-based
materials hold promise as injectable carriers of MSCs for orthopaedic tissue engineering applications.
Table 5.1: Change in pH (from control) of individual initiator components and their combinations over time. Data are presented as means ± standard deviation, n = 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔpH at 0 min</th>
<th>ΔpH at 2 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>-6.88 ± 0.02</td>
<td>-6.49 ± 0.06</td>
</tr>
<tr>
<td>100 mM</td>
<td>-3.89 ± 1.37</td>
<td>-4.08 ± 1.33</td>
</tr>
<tr>
<td>10 mM</td>
<td>-0.05 ± 0.12</td>
<td>-0.39 ± 0.08</td>
</tr>
<tr>
<td>NaPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>-0.08 ± 0.01</td>
<td>-0.63 ± 0.02</td>
</tr>
<tr>
<td>100 mM</td>
<td>-0.06 ± 0.03</td>
<td>-0.34 ± 0.06</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.06 ± 0.03</td>
<td>-0.14 ± 0.07</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>-5.05 ± 0.01</td>
<td>-4.84 ± 0.01</td>
</tr>
<tr>
<td>100 mM</td>
<td>-3.96 ± 0.05</td>
<td>-4.14 ± 0.05</td>
</tr>
<tr>
<td>10 mM</td>
<td>-1.03 ± 0.04</td>
<td>-0.66 ± 0.03</td>
</tr>
<tr>
<td>Asc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>0.13 ± 0.03</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>100 mM</td>
<td>-0.03 ± 0.11</td>
<td>-0.03 ± 0.11</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.06 ± 0.06</td>
<td>-0.01 ± 0.09</td>
</tr>
<tr>
<td>Asc-2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
<td>0.13 ± 0.09</td>
<td>0.04 ± 0.06</td>
</tr>
<tr>
<td>10 mM</td>
<td>0.17 ± 0.01</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>APS/AA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>-6.38 ± 0.19</td>
<td>-7.07 ± 0.09</td>
</tr>
<tr>
<td>100 mM</td>
<td>-5.43 ± 0.19</td>
<td>-6.63 ± 0.08</td>
</tr>
<tr>
<td>10 mM</td>
<td>-1.14 ± 0.16</td>
<td>-1.50 ± 0.18</td>
</tr>
<tr>
<td>NaPS/Asc*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 mM</td>
<td>-6.16 ± 0.05</td>
<td>-6.74 ± 0.02</td>
</tr>
<tr>
<td>100 mM</td>
<td>-4.82 ± 0.02</td>
<td>-6.08 ± 0.01</td>
</tr>
<tr>
<td>10 mM</td>
<td>-0.92 ± 0.04</td>
<td>-1.06 ± 0.04</td>
</tr>
<tr>
<td>NaPS/Asc-2*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
<td>-0.04 ± 0.02</td>
<td>-2.37 ± 0.03</td>
</tr>
<tr>
<td>10 mM</td>
<td>-0.08 ± 0.04</td>
<td>-0.28 ± 0.01</td>
</tr>
</tbody>
</table>

*Asc-2 soluble at 100 and 10 mM only.
*For the combinations, concentrations represent the final concentration of each of the initiator components in solution.
**FIGURE 5.1:** Chemical structure of the species involved in OPF crosslinking as examined in this study. Ammonium persulfate (APS) or sodium persulfate (NaPS) were combined with one of the ascorbate derivatives: ascorbic acid (AA), sodium ascorbate (Asc), or magnesium ascorbate-2-phosphate (Asc-2) to initiate the crosslinking reaction.
**Figure 5.2:** pH change from control and corresponding marrow stromal cell viability (fraction live cells normalized to positive control) for initiator components and their combinations (10-500 mM). (A) Viability and pH change examined after 2 hours continuous exposure to reagents. (B) Viability examined after 2 hrs, with 10 min exposure to reagents. pH values were determined at 0 min for the individual components and 10 min for the combinations. Error bars represent standard deviation; n = 3.
**Figure 5.3:** Fluorescent microscopy images of marrow stromal cells after 2 hrs continuous exposure to initiator components and treated with LIVE/DEAD reagent. (A) Live (positive) control. (B) Dead (negative) control. (C) Exposure to APS (100 mM). (D) Exposure to AA (100 mM). (E) Exposure to Asc (100 mM). (F) Exposure to Asc-2 (100 mM). (G) Exposure to APS/AA (100 mM each component). (H) Exposure to NaPS/Asc-2 (100 mM each component). Scale bar indicates 200 μm, magnification 100X.
Figure 5.4: Time to onset and completion of gelation as assessed by rheometry. (A) Gelation times for the APS/AA and NaPS/Asc-2 initiator systems (10 and 100 mM) using OPF 10K. (B) Gelation times for the APS/AA and NaPS/Asc-2 initiator systems (10 and 100 mM) using OPF 1K. Error bars represent standard deviation; n = 3.
CHAPTER VI

EFFECT OF SWELLING PROPERTIES OF OLIGO(POLY(ETHYLENE GLYCOL) FUMARATE) HYDROGELS AND ADDITION OF MEDIA SUPPLEMENTS ON IN-VITRO OSTEOGENIC DIFFERENTIATION OF ENCAPSULATED MARROW STROMAL CELLS

ABSTRACT

The novel polymer, oligo(poly(ethylene glycol) fumarate) (OPF), crosslinked with a redox radical initiation system has recently been developed in our laboratory as an injectable, biodegradable cell carrier for regeneration of orthopaedic tissues. Hydrogels prepared from OPF with poly(ethylene glycol) of two different chain lengths exhibited significantly different swelling characteristics after crosslinking (fold swelling: 17.5±0.2 vs. 13.4±0.4). The effects of these differences on the differentiation of encapsulated rat marrow stromal cells (MSCs, seeding density ~14 million cells/mL) cultured for 28 days both with and without osteogenic supplements (dexamethasone) were determined via histology and biochemical assays for alkaline phosphatase, osteopontin and calcium. Evidence of MSC differentiation in these samples (6 mm diameter X 0.5 mm thick before swelling) was apparent over the culture period for samples without dexamethasone, but there was large variability in calcium production between constructs using cells of the same source. Differentiation was also seen in samples cultured with osteogenic supplements, but calcium deposition varied depending on the source pool of MSCs. By day 28, osteopontin and calcium results suggested that, in the presence of dexamethasone, OPF hydrogels with greater swelling promoted embedded MSC differentiation over those that swelled less (43.7±16.5 µg calcium/sample and 16.4±2.8 µg calcium/sample, respectively). In histological sections, mineralized areas were
apparent in all sample types many microns away from the cells. These experiments strongly support the usefulness of OPF hydrogels as injectable MSC carriers for bone regeneration and indicate that hydrogel swelling properties can influence differentiation of encapsulated progenitor cells.

**INTRODUCTION**

In the past decade, research in the area of orthopaedic tissue engineering has endeavored to produce methods that restore defects in bone and cartilage. One such approach is to deliver cells to the site of injury and, thus, promote localized healing of the desired tissue. In order to reduce patient discomfort, an ideal cell carrier would be both injectable and biodegradable, so that cells could be introduced via minimally-invasive means and a second surgery would not be required to remove the carrier material [149]. As outlined previously, a suitable injectable material should be biocompatible, both in regards to the resident cells and surrounding tissue, have biocompatible degradation products, demonstrate appropriate degradation times and mechanical properties to protect the defect site while promoting tissue growth, be easily sterilizable, and exhibit clinically-relevant setting times and handling properties [149]. Additionally, materials to be used as cell carriers (either with or without macroporosity) should have diffusional properties sufficient to allow nutrient exchange throughout the construct.

While natural injectable materials including fibrin [58, 124] and alginites [60, 125] have been examined as hydrogels for cell encapsulation, concerns with possible immune rejection and availability of large quantities of natural materials have led others to study synthetic injectable materials as cell carriers [149]. These include materials based on acrylamides [128], poly(vinyl alcohol) [150], and poly(ethylene glycol) (PEG)
[63, 74, 151]. The advantage of these hydrogel materials is that they have a high degree of swelling in aqueous environments and thus can promote viability of cells in constructs with a thickness of several millimeters [74] and, through altering synthesis parameters, their mechanical and degradative properties can be tailored for specific applications [152].

Our laboratory has developed a novel injectable synthetic hydrogel material, oligo(poly(ethylene glycol) fumarate) (OPF), for orthopaedic tissue engineering applications. This material meets many of the requirements detailed above for an ideal injectable cell carrier. Hydrolysis of the ester bonds in the OPF backbone results in biodegradation of the resulting crosslinked hydrogels [115]. The cytocompatibility of the linear OPF molecule and leachable fractions from the crosslinked hydrogels have been demonstrated in vitro using rat marrow stromal cells (MSCs) [131]. In addition, evidence of the biocompatibility of pre-crosslinked gels from various OPF formulations has been observed in vivo in rabbits [115]. Depending on the selection of crosslinking molecules, the mechanical and degradative properties of these hydrogels may be altered as required for given applications [115, 116, 148].

A cytocompatible, water-soluble radical initiation system, ammonium persulfate/N,N,N',N'-tetramethylethylenediamine (APS/TEMED), has recently been identified for this oligomer, allowing for the encapsulation of rat MSCs at 37°C within 10 min [153]. Thus, cell-OPF hydrogel constructs can be formed on a clinically-relevant time scale, and the spatial distribution of the encapsulated cells can be controlled. Additionally, the MSC-OPF solution is easily drawn into syringe at room temperature, making the system relatively easy to manage in a clinical setting. In particular, while
many previous PEG-based materials for cell encapsulation have involved
photocrosslinking to form hydrogels [74, 151, 152], in this case, gelation is based only on
changes in temperature, which may be especially advantageous in areas where light
penetration is limited.

Since MSCs have demonstrated the potential to differentiate into multiple cell
types [154], the use of OPF as a carrier for MSCs is envisioned to aid regeneration of a
variety of orthopaedic tissues. However, this study focuses only on the osteogenic
differentiation of encapsulated rat MSCs. Specifically, these experiments were designed
to characterize the gelation time and swelling properties of OPF hydrogels using two
different OPF formulations (OPF 10K and OPF 3K) crosslinked with PEG-diacrylate
(PEG-DA). Subsequently, the effects of these two OPF formulations on the
differentiation of encapsulated rat MSCs cultured both with and without osteogenic
supplements (dexamethasone) were determined via histology and biochemical assays for
matrix molecules associated with bone.

**MATERIALS AND METHODS**

**pH Experiments**

Solutions of 25 mM APS (EM Science, Gibbstown, NJ), 25 mM TEMED (Sigma-
Aldrich, St. Louis, MO) as well the combination APS/TEMED (25 mM each
component), were prepared in triplicate in MSC culture media (DMEM high glucose
(Gibco, Grand Island, NY) supplemented with 10% v/v fetal bovine serum (Gemini,
Calabasas, CA), 10 mM β-glycerophosphate, 50 mg/L ascorbic acid, 250 µg/L fungizone,
100 mg/L ampicillin and 50 mg/L gentamicin (all from Sigma-Aldrich)). The pH of these
solutions was recorded immediately after mixing with a digital pH meter (AP5, Fisher
Scientific, PA). The pH change in reference to media containing no initiators was then calculated [155].

**OPF Synthesis and Characterization**

Two formulations of OPF were synthesized from PEG of different nominal number average molecular weight: 10,000 g/mol (designated OPF 10K) or 3,300 g/mol (OPF 3K), following established procedures [77, 116]. After synthesis, the resulting macromer was purified via recrystallization from ethyl acetate (Fisher Scientific, Pittsburgh, PA) and precipitation with ethyl ether (Fisher Scientific). The resulting powder was vacuum dried at <5 torr and stored at -20°C until use. The OPF was then characterized via gel permeation chromatography (GPC, Waters Model 410, Milford, PA, column 50-100,000 Da range). The molecular weight of both the PEG starting material and the resulting OPF was evaluated (3 samples of each type used).

**Rheometry Experiments**

OPF 10K or OPF 3K and the crosslinker PEG-DA (nominal MW 3400, Nektar Therapeutics, Huntsville, AL) were dissolved in phosphate-buffered saline (PBS, Gibco) (25 wt% initial polymer, polymer weight ratio: 2 OPF: 1 PEG-DA) [116, 156]. The initiator combination APS/TEMED (final concentration of each initiator component was 25 mM) was then added. The modulus of the complex viscosity ($\eta^*$) of these solutions was monitored via a dynamic oscillatory test at 37°C using a rheometer (Rheolyst AR1000, TA Instruments, New Castle, DE). Gelation onset was determined to be the first time at which there were at least 4 consecutive points with $\geq 20\%$ change in complex viscosity modulus. Gelation completion was considered the first time at which there
were at least 4 consecutive points with less than 1\% change in $\eta^*$ modulus [155].

Samples were run in triplicate.

**OPF Hydrogel Swelling Experiments**

Following previously developed methods for determination of hydrogel swelling ratio [116, 156], OPF was crosslinked as described for the rheometry experiments, except using distilled, deionized water (ddH$_2$O) instead of PBS. Once mixed, the solutions were injected into Teflon molds with a thickness of 1 mm and then placed at 37°C for 8 min. After crosslinking, the hydrogels were cut into discs with a cork-borer (13 mm diameter). The resulting discs were weighed immediately ($W_i$), swollen in ddH$_2$O, weighed ($W_s$), and then dried at <1 torr and weighed again ($W_d$). Fold swelling and theoretical sol fraction were then calculated for both OPF types ($n = 4-5$ per treatment), using the following equations:

\[
\text{Fold Swelling: } \frac{W_s - W_d}{W_d}
\]

\[
\text{Sol Fraction: } \frac{0.25W_i - W_d}{0.25W_i}.
\]

**Marrow Stromal Cell (MSC) Isolation and Preculture**

MSCs were isolated from male Wistar rats (~150 g) as previously described [44]. Briefly, femurs and tibias were excised from the rats under aseptic conditions and the marrow was flushed from the bone with cell culture media (same as that used for preculture, see below). The cells were then seeded in T-75 culture flasks and precultured for 1 wk before use in this study. Non-adherent cells were removed and the media was changed after days 1 and 4 in culture. Four cell isolation procedures were performed, each time using 3 rats. Two of these isolations (I+1 and I+2) were precultured in media
containing dexamethasone, known to encourage osteoblastic differentiation of rat MSCs (media as described for pH study, with the addition of 10⁻⁸M dexamethasone (Sigma-Aldrich)) while the other two isolations (I-1 and I-2) were precultured in identical media without dexamethasone.

**MSC Encapsulation and Culture**

At the end of the preculture period, a combination of OPF 10K or 3K (0.1 g) was combined with PEG-DA (0.05 g) and sterilized via exposure to UV light for 3 hrs. Then, 300 µl of sterilized PBS was added and the polymer was allowed to dissolve in an incubator. Concentrated solutions of 300 mM APS and TEMED were also prepared in PBS and filter-sterilized. The expanded MSCs were then exposed to trypsin-EDTA (0.25%, Sigma-Aldrich), resuspended in PBS and counted with a cell-size analyzer (Beckman-Coulter, Fullerton, CA).

To begin the encapsulation process, 46.8 µL of the APS solution, followed by 46.8 µL of the TEMED solution was added to the OPF solution and mixed. Next, the cell suspension (168 µL containing 7 million cells) was added to the OPF solution and gently agitated. The polymer/cell mixture was loaded in a syringe and quickly injected in pre-sterilized Teflon molds (6 mm diameter by 0.5 mm thick). After 8 min in an incubator, the newly-formed gels were aseptically transferred to 12-well plates and 2.5 mL of the appropriate media was added. Over the following 3 hrs, the media was changed twice to assure the removal of unreacted hydrogel components.

During the 28-day culture period, the media was changed every 2-3 days. After 1, 7, 14, 21 and 28 days of culture, samples were removed for biochemical (n=4, except day 28 OPF 3K without dexamethasone n=3) and histological analysis (n=3). Media was
collected at every media change from the 28-day samples and stored at $-20^\circ$C for later analysis for osteopontin secretion (n=3). Samples containing cells from I-1 and I+1 were used for time points representing days 1, 7, 14 and 21, while samples containing cells from I-2 and I+2 were used for time points representing days 1, 7, 21 and 28.

In addition, OPF 10K and 3K hydrogels without cells were made as described above, except with the addition of PBS instead of the cell suspension. These blank hydrogels, cultured in media with and without dexamethasone, were subject to biochemical analysis at the same time points as the samples containing MSCs.

**OPF Hydrogel Degradation Experiments**

Identical samples to the blank hydrogels described above were fabricated for a 28-day degradation study with the same time points as those used for the biochemical assays. The samples were kept in an incubator in primary media (DMEM with 10% fetal bovine serum and antibiotics, but without other supplements), with media changes every 2-3 days. At the appropriate time points, fold swelling and sol fraction were calculated as detailed previously (n=3-4 per time point).

**Biochemical Assays for MSC Differentiation**

At the given time points, the samples and hydrogel blanks were removed from culture, rinsed in PBS and homogenized with a pellet grinder (Fisher Scientific) in 500 μL ddH$_2$O. The samples were stored at $-20^\circ$C until just before analysis, when they were subjected to 3 freeze-thaw cycles including sonication with ice for 30 min after each cycle. Except for the osteopontin assay, each sample (n=3-4 per treatment per assay per time point) was run in triplicate. In the case of osteopontin, each sample was run in duplicate.
Cell Number

Homogenates underwent analysis for double-stranded DNA content using the PicoGreen assay (Molecular Probes, Eugene, OR) as per manufacturer’s instructions [157]. 43 μL of each sample was combined with 107 μL of supplied buffer and 150 μL of PicoGreen dye in a 96-well plate. Standards of bacteriophage λ-DNA were prepared in concentrations of 0-0.5 μg/mL and used as a reference. The plate was read on a fluorescent plate reader (Bio-tek Instruments, Winooski, VT) with excitation/emission wavelengths of 485/528. The fluorescence of blank gels was subtracted to determine the amount of DNA/sample from the standard curve.

Known numbers of MSCs from multiple isolations were also analyzed with this method to determine the amount of DNA/cell (3.46 pg/cell), which was used subsequently as a conversion to cell number for each sample.

Alkaline Phosphatase (ALP)

Amount of alkaline phosphatase present in each homogenized sample was determined using Sigma Diagnostic Kit #104, according to manufacturer’s instructions [157]. 60 μL of each sample was combined with 40 μL of alkaline buffer and 100 μL of substrate in a 96-well plate. After an 1-hr incubation at 37°C, the reaction was stopped with 100 μL of 0.3 N sodium hydroxide (Fisher Scientific) and the absorbance was read at 405 nm on a plate reader (Powerwave X, Bio-tek Instruments). Absorbance of the samples was adjusted with blank controls. p-Nitrophenol standards (supplied) in concentrations from 0-300 μM were used as a reference to calculate the ALP activity per hour.
Osteopontin (OPN)

Media samples were analyzed for osteopontin content via a sandwich immunoassay (Assay Designs, Ann Arbor, MI), as per manufacturer’s instructions. Media from samples both with and without MSCs were pooled to represent 1-7 days, 8-14 days, 15-20 days, and 21-28 days in culture [157]. These media samples were added to 96-well plates that had been pre-coated with a rabbit polyclonal antibody to rat osteopontin. Rat OPN standards included with the kit were used as reference. After addition of a secondary antibody (rabbit) labeled with horseradish peroxidase and appropriate substrate, the absorbance at 450 nm was recorded with a plate reader (Powerwave X, Bio-tek Instruments). Samples from blank hydrogels showed no sign of OPN content, so no adjustment to the absorbance of the remaining samples was necessary.

Calcium

Prior to analysis of calcium content for each homogenate, 0.5 N acetic acid (Fisher Scientific) was added to the samples and they were placed on a shaker table overnight to dissolve mineral deposits. Calcium amount/sample was determined using Sigma Diagnostic Kit #587, according to manufacturer’s instructions [157]. 10 μL of each sample was mixed with 300 μL of combined binding and buffer reagents in a 96-well plate. Standards prepared from CaCl₂ (Sigma-Aldrich) in concentrations from 0-200 μg/mL were used as a reference. After 10 min at room temperature, the absorbance was read at 575 nm on a plate reader (Powerwave X, Bio-tek Instruments) and the samples were adjusted with blank controls prior to calculation of the calcium content.
**Histology**

After culture, histology samples were rinsed in PBS, fixed in 10% neutral buffered formalin (Sigma-Aldrich) and serially dehydrated in ethanol. The discs were then embedded in paraffin and 12 µm-thick cross-sections were cut on a microtome (Microm, Walldorf, Germany). The sections were subsequently stained with Von Kossa reagent (5% w/v, Sigma-Aldrich) and exposed to direct sunlight for 30 min to visualize the mineralized matrix (black) and counter-stained with Safranin-O (0.5% w/v, Sigma-Aldrich; cells and polymer appear red). The resulting slides were imaged with a microscope (Eclipse E600, Nikon, Melville, NY) and attached video camera (3CCD Color Video Camera DXC-950P, Sony, Park Ridge, NJ).

**Statistical Analysis**

Data from all studies were analyzed using ANOVA and Tukey’s Multiple Comparison Test or an unpaired Student’s t-test (p ≤ 0.05). The results for the DNA, ALP and calcium assays were log-transformed prior to statistical analysis due to large variances in the data. Results are reported as mean ± standard deviation.

**RESULTS**

**pH Experiments**

Results of the pH study can be found in Table 6.1. After combining the initiator components, the resulting pH was within 1 unit of the control media.

**OPF Synthesis and Characterization**

Results of the GPC analysis of the synthesized OPF macromers is found in Table 6.2.
**Rheometry Experiments**

Results detailing the times to onset and completion of gelation for the OPF 10K and 3K appear in Table 6.3. Statistical analysis revealed that the two formulations had the same onset time, but that the OPF 10K finished crosslinking in a significantly shorter time than the OPF 3K.

**OPF Hydrogel Swelling Experiments**

Results showing fold swelling and sol fraction for OPF 10K and 3K hydrogels are found in Table 6.4. While there was statistically equivalent sol fractions for each formulation, the OPF 10K samples swelled significantly more than the OPF 3K.

**OPF Hydrogel Degradation Experiments**

Fold swelling and sol fraction of OPF 10K and 3K hydrogels over time are found in Figure 6.1. There was no statistical difference in swelling for the OPF 10K hydrogels over the 28-day period. However, there was a significantly larger fold swelling for days 21 and 28 than for days 1 and 7 with the OPF 3K hydrogels. Also for this formulation, there was significantly greater swelling at day 28 than day 14. Statistical analysis further revealed that the OPF 10K hydrogels had a significantly smaller sol fraction on days 1 and 7 than on days 14 and 21, while there was no difference in sol fraction over time for the OPF 3K hydrogels.

**Biochemical Assays for MSC Differentiation**

For ease of comparison, in the following text, the treatments used in this study will be referred to as 10K- (OPF 10K hydrogel cultured without dexamethasone), 3K- (OPF 3K hydrogel cultured without dexamethasone), and 10K+ and 3K+ (corresponding hydrogels cultured with dexamethasone).
Cell Number

Cell number/sample at each time point is depicted in Figure 6.2. While a general trend of decreasing cell number/sample seems apparent, a statistically significant decrease in cell number was found only in the 10K+ day 28 samples as compared to the rest of the time points for that treatment. Additionally, at day 28, the 10K+ samples were determined to have significantly fewer cells than the other treatments.

Alkaline Phosphatase

ALP results over time are presented in Figure 6.3. Statistical analysis revealed significantly higher ALP values for the 10K+ and 3K+ samples at day 28 than the rest of the time points. There was no significant change in ALP for the non-supplemented samples over the culture period. At day 1, the 10K- constructs demonstrated significantly higher ALP levels than the 3K+.

Osteopontin

Results of the OPN immunoassay are found in Figure 6.4. A statistically significant increase in total OPN secretion was seen with each time point for the 10K- samples, while there was no difference in total OPN over time for the 3K- treatment. OPN amounts at day 7 were significantly lower than at day 20 or 28 for the 10K+ constructs, but there was no significant increase in levels from days 14-28. Similarly, for the 3K+ samples, there was a significant increase in total OPN up to day 21, but the day 20 and 28 levels were found to be statistically equivalent.

At all time points tested (days 7, 14, 20 and 28), the same statistical trends were found between treatments. There was no significant difference between the OPN levels of the 10K- and 3K- samples, and the non-supplemented samples always exhibited lower
OPN amounts than either the 10K+ or the 3K+. In addition, the 10K+ constructs demonstrated greater OPN secretion than the 3K+ constructs at all time points.

**Calcium**

Calcium content of the constructs at various time points is presented in Figure 6.5. For the both the 10K- and 3K- samples, there was significantly more calcium deposition at day 28 than at the rest of the time points. The calcium levels for the 10K+ constructs were significantly higher at days 21 and 28 than at days 1 and 7. Additionally, there was a significant increase in calcium between days 14 and 21. However, there was no difference in calcium levels between days 21 and 28 for this treatment. There was also no significant change in calcium for the 3K+ constructs over the culture period.

The data were further examined at days 21 and 28, when significant calcium deposition had occurred for many sample types. At day 21, the 10K+ constructs were found to have produced more calcium than the other samples. There was no significant difference in calcium level between the 3K- and 3K+ samples at this time point. At day 28, there was no significant difference in calcium amount between the 10K- and 3K- samples, and there was no difference between levels for these samples and that of the 10K+. The 10K- produced more calcium than the 3K+ constructs, and the 3K+ calcium levels were significantly lower than for the 3K- samples. The 10K+ constructs exhibited a significantly higher calcium content than the 3K+ constructs at this time point.

**Histology**

Composite images showing representative cross-sections from all treatments at days 7 and 21 of *in-vitro* culture are found in Figure 6.7. Figure 6.8 depicts similar images from day 28. In these specimens, the polymer (labeled P) appears red-pink, non-
mineralized cells are red, and mineralized matrix (labeled M) appears brown-black. Arrows indicate the location of cells, with or without associated mineral. Histological processing resulted in tearing of some of the samples, which appear as holes (white) in the images. In addition, folding of specimens containing mineralized matrix occurred during processing, resulting in wavy edges with these samples.

Qualitative evidence of the formation of mineralized matrix is found in the dark staining of many cells (and in some cases the entire hydrogel) for the samples with osteogenic supplements at day 21, as compared to the red cells seen at day 7 (Figure 6.7 A-B, D-F). In contrast, there is minimal appearance of mineral formation in samples cultured without supplements at this time point (Figure 6.7 C). Additionally, mineralized matrix is observed not only directly adjacent to the cells, but in areas microns away, for all sample types by day 28 (Figure 6.8). Figure 6.8 also confirms the variability in calcium deposition for samples grown without dexamethasone, quantified previously in the calcium bioassay (6.8 A-D).

Although not apparent in these images, the hydrogels seemed to have more mineral in center than at the edges. For the samples cultured without osteogenic supplements, there appeared to be bands of lighter and heavier mineral deposits in many of the more mineralized specimens (Figure 6.8 D). This was most evident macroscopically during culture as swirls of more opaque areas, contrasting with areas that were more transparent. The blank gels remained transparent throughout culture. Mineral deposition seemed more homogenous throughout the supplemented samples, but in some cases (10K+, I+2), a line of mineral appeared to be moving longitudinally throughout the gel with time (Figures 6.7 E and 6.8 E). Although these upper and lower
faces of these specimens were not marked before cutting, the top face (exposed to media) of these samples did appear more opaque than the bottom (facing base of cell culture well) when they were removed at day 28.

**DISCUSSION**

This study was designed to assess the effects of material properties of OPF hydrogels on the osteogenic differentiation of embedded MSCs cultured *in vitro* with and without osteogenic supplements (dexamethasone). We have found in previous studies that, by changing the molecular weight of the PEG used in oligomer synthesis, the swelling and mechanical properties of crosslinked OPF hydrogels can be altered [148, 155, 156]. Therefore, for this study, two OPF formulations (OPF 10K and 3K, see Table 6.2) that, in the presence of the APS/TEMED initiator system and a bis-acrylamide crosslinker, resulted in hydrogels with different swelling properties [148] were chosen to encapsulate rat MSCs.

**pH, Rheometry, Swelling and Degradation Studies**

These experiments were undertaken to characterize the OPF-based hydrogels prior to their use in cell encapsulation studies. In previous studies in our laboratory, the pH of the initiator combination was determined be a concern in cell-encapsulation procedures, as these components are in direct contact with MSCs during the crosslinking reaction [155]. However, pH results in cell culture media indicate that this combination remained near neutral after mixing (Table 6.1), which may help explain its cytocompatibility, as demonstrated in preliminary work with OPF 10K hydrogels [153]. Also from previous work, it was ascertained that short crosslinking times are important to minimize length of cell exposure to any cytotoxic agents that may be included in the
crosslinking formulation [153, 155]. Since different OPF types can have different
crosslinking times [155], rheometry experiments were used to determine gelation times of
OPF 10K and 3K crosslinked with PEG-DA of a suitable molecular weight (3400), as
determined in prior cytocompatibility studies [131].

While different gelation completion times were found for the two formulations
(Table 6.3), both demonstrated gelation onset within 4 min at 37°C. Thus, 8 min was
chosen for the encapsulation procedure in order to form a solid network, but minimize
MSC contact with crosslinking components. However, since swelling and mechanical
properties of similar gels were influenced by crosslinking time [148], it was important to
assure that, after 8 min at 37°C, differences in hydrogel swelling were apparent between
the OPF formulations used in this study. Results showed that the OPF 10K hydrogels
had a statistically larger fold swelling, indicative of a larger hydrogel mesh size [79, 116],
than the OPF 3K. In this experiment, ddH₂O was used as the swelling media to facilitate
comparison with previous results, but similar fold swelling values were also obtained in
cell culture media (see Figure 6.1).

In cell encapsulation studies for cartilage tissue engineering, rate of hydrogel
degradation was found to influence the type and location of generated extracellular
matrix (ECM) [152, 158]. Therefore, the degradation of OPF 10K and 3K hydrogels
under cell culture conditions was monitored over 28 days (Figure 6.1). The results
demonstrated that there was no change in swelling properties for the OPF 10K over this
time period and, while statistically significant, the fold swelling between day 1 and day
28 for the OPF 3K increased only from 12.7 ± 0.5 to 14.2 ± 0.6. This, combined with the
observation that the gels remained intact throughout the experiment indicates that
hydrogel degradation was not a major contributor to MSC differentiation/ECM production as observed in this study.

**Biochemical Assays for MSC Differentiation**

The samples formed from OPF 10K and 3K with embedded MSCs were subsequently cultured with and without the addition of dexamethasone, a synthetic glucocorticoid known to induce osteogenic differentiation of MSCs in both rats and humans [146, 159-162]. β-glycerophosphate was included in both types of media to accelerate mineralization [163]. At each time point, the constructs were analyzed for cell number, ALP activity, OPN secretion and calcium content.

In these constructs, although there seemed to be a trend of decreasing cell number over time, this was only significant with the 10K+ samples at day 28 (Figure 6.2). While the APS/TEMED initiator system has been shown in previous work with OPF to be cytocompatible [153], some cell death may occur over the first few days as a result of the encapsulation procedure. At later time points, the increase in mineralized matrix may hinder diffusion of the DNA out of the gel, and thus reduce its availability for the bioassay. Alternatively, the accumulation of matrix may prevent nutrient transport to the cells and cause further cell death with time. A similar apparent decrease in viability over time was seen with rat calvarial osteoblasts embedded in PEG-DA hydrogels [151]. In any case, there is no evidence of MSC proliferation after encapsulation in either OPF 10K or 3K hydrogels.

ALP, considered an early marker for osteoblastic differentiation, is a cell-membrane associated phosphatase thought to be involved in the onset of ECM mineralization [163, 164]. However, in these experiments, there was little difference in
ALP content over time, with a significant increase noted only at day 28 for 10K+ and 3K+ samples (Figure 6.3). It could be possible that a peak occurred between time points, or that the temporal expression of this marker was altered due to the encapsulation process. Unlike in other systems studying osteogenic differentiation, after encapsulation, there was minimal MSC proliferation, and it has been suggested by experiments with osteoblasts on tissue culture polystyrene (TCPS) that the upregulation of messenger RNA (mRNA) for ALP is connected to the termination of the proliferation stage [163]. However, it is also important to consider that, since this molecule is bound to the cell membrane, there may be problems in extracting it from the gels for analysis, leading to an inability to distinguish trends in expression.

In contrast to ALP, OPN is a secreted marker of osteoblastic differentiation. OPN is a glycoprotein known to possess a cell-binding domain, as well as possible calcium-binding sites [163, 164]. Although the exact temporal expression of this protein may vary, OPN levels increase just prior to or with the onset of mineralization [163, 165, 166]. In this assay, cell culture media from the different sample types was pooled to represent OPN secretion from days 1-7, 8-14, 15-20 and 21-28. While this was done to facilitate comparison with the other markers at the same time points, it may decrease the ability to determine exactly when peak OPN secretion occurs in this system. The general shape of the cumulative release curves (Figure 6.4) were similar for the two construct types cultured with supplements. While OPN secretion in the supplemented samples decreased after days 14-21, this was not true for the 10K- samples. The addition of dexamethasone to the culture media immediately after cell isolation could have selected a sub-population of cells that demonstrated higher OPN production. It should be noted,
however, that while cells from the same isolation (I-2) were used for the 10K- and 3K-samples, this was a different isolation than those used for the 10K+ and 3K+ samples (I+2).

At all time points, the samples cultured with supplements produced higher levels of OPN than those cultured without, indicative of further differentiation towards the osteoblastic phenotype with the addition of dexamethasone. While there was no increase in rate of OPN secretion over time in the supplemented samples, there was a decrease after a certain point. Due to the lack of proliferation in this system, the timing of OPN upregulation may have been shifted, or, at later time points, OPN could be increasingly sequestered in the mineralizing ECM and thus not available for assay in the media. In addition, the reports of temporal distribution of osteoblastic markers have been, in some cases, based on mRNA upregulation [163], which may not correspond to final, active protein amounts. This is particularly true for OPN, where not only production, but subsequent phosphorylation is important for its role in matrix mineralization [167, 168].

Additionally, for the constructs cultured in supplemented media, the OPF 10K hydrogels resulted in higher OPN levels than the 3K at all time points. It is not clear from this assay whether this hydrogel formulation encourages production, or just secretion, of OPN, since the OPF 10K hydrogel swells more than the 3K and thus may allow better diffusion of OPN into the media. However, that this trend was found only with 10K+ (rather than all 10K) samples, suggests that the differences in OPN levels cannot be explained solely by increased diffusion.

Calcium, a late marker for osteogenic differentiation [163, 166], was found only in day 21 and 28 samples (Figure 6.5). Blank hydrogels showed no significant calcium
deposition at any time point, so calcium accumulation in these constructs was not due to passive precipitation from the media. Large variability was found in 10K+ samples on day 21 and 10K- and 3K- on day 28, which can, in part, be explained by tracing the source of the MSCs used for these samples.

In this study, 4 isolations from 3 rats each were used, assuming that the pool of donors for each isolation was large enough to mitigate the effects of differences in osteoprogenitor cells between animals. This was found not to be the case. Considering only the OPF 10K samples, at day 21, the 10K- contained two samples with cells from I-1 and two from I-2 (see Figure 6.6 A), while the 10K+ included samples with cells from I+1 and I+2 (Figure 6.6 B). Although the response from I-1 and I-2 cells was similar at this time point, there was a large difference in calcium deposition between I+1 and I+2 (I+1 average: 350 µg/sample; I+2 average: 23 µg/sample). In contrast, samples from day 28 contained only cells from harvest I-2 or I+2 (see Figure 6.6). Here, there was little variation between samples grown in supplements, but constructs cultured without supplements demonstrate wide variability in calcium content.

When rat MSCs were cultured on TCPS, a subpopulation of cells that could form mineralized nodules without the presence of dexamethasone was identified, but the addition of dexamethasone increased the amount of these nodules [159]. Due to randomized cell seeding in this study, it could be possible for more of these dexamethasone-independent cells to be placed in one hydrogel than another, accounting for this varied response. The presence of osteogenic supplements, which increased the number of nodules formed from the same amount of progenitors [159], could mitigate the heterogeneity of the cell population within a single isolation. However, for the 10K+
constructs, each isolation as a whole (I+1 or I+2) seemed to differentiate on a different time scale. (There was not difference in calcium content over time for the 3K+ scaffolds with cells from either isolation.) These effects can be reduced in future experiments by using a larger pool of donors, or screening for osteogenic potential before encapsulation.

At both 21 and 28 days, more calcium was produced in the 10K+ than the 3K+ hydrogels. This, in conjunction with OPN results, suggests that the OPF 10K hydrogel promotes MSC differentiation in the presence of osteogenic supplements. This may be due to a higher fold swelling ratio, leading to enhanced diffusion of growth factors, nutrients and/or mineralized matrix components throughout these hydrogels.

**Histology**

Images from cross-sections of these constructs confirm the variability of calcium deposition for both day 21 and day 28 samples. In all cases, there is evidence of some mineralized (dark) cells by day 21. In the 10K+ (I+1) constructs, there is mineralized matrix throughout the entire hydrogel at this time point, with only a few non-mineralized cells (Figure 6.7 D and G). This treatment using cells from I+2 also shows evidence of mineral deposition, but only partially filling the gel (Figure 6.7 E and H). The 3K+ samples exhibit only a few areas of localized mineralization at day 21 (Figure 6.7 F and I).

At day 28, the 10K+ (I+2) specimens demonstrate what appears to be darker and more extensive Von Kossa staining than at day 21, indicating that these cells, while not responding as early as the I+1 cells, continued to produce mineralized matrix over time (Figures 6.7 E and 6.8 E). Some of the samples cultured without dexamethasone had mineralized deposits filling the hydrogel, while others stained very lightly, further
confirming data from the calcium assay (Figure 6.8 A-D). With all samples at this time point, there is evidence of mineralized bands not only adjacent to cells, but throughout the hydrogel. This level of mineralization has not been previously reported for encapsulated cells cultured in vitro. When rat calvarial cells were embedded in PEG-DA hydrogels with cell-adhesion peptides, mineralized matrix appeared only in areas directly adjoining the cells [151].

Possible explanations for the accumulation of mineral throughout the OPF hydrogels include enhancement of the homogeneous nucleation of calcium-phosphate crystals [164, 169]. In vivo, the lumen of matrix vesicles may contain artificially high levels of calcium and phosphate ions to induce crystal nucleation [164, 169]. The OPF hydrogel could allow the migration of these vesicles away from the cells that created them, or may act in an independent manner to locally increase the concentrations of these ions. Alternatively, the OPF hydrogel could act to promote heterogeneous nucleation by allowing secreted extracellular matrix molecules, such as bone sialoprotein, to attain the proper conformation to enhance crystal growth [164, 167, 169]. In addition, secondary nucleation is possible, with growth of new crystals beginning on the faces of those previously formed [169].

CONCLUSIONS

These studies demonstrated that OPF hydrogels can be crosslinked with PEG-DA and the initiators APS/TEMED for 8 min at 37°C to produce hydrogels with different swelling properties. The effect of these properties on osteogenic differentiation of encapsulated rat MSCs with and without the presence of dexamethasone was then investigated. Evidence of MSC differentiation, including OPN and calcium results, was
apparent over the culture period for samples without dexamethasone, but there was large variability in calcium production between constructs with cells of the same source. Differentiation was also seen in samples cultured with osteogenic supplements, but calcium deposition varied depending on the source pool of MSCs. By day 28, OPN and calcium results suggested that, in the presence of dexamethasone, OPF 10K hydrogels promoted embedded MSC differentiation over OPF 3K hydrogels. In histological sections, mineralized areas were apparent for all sample types many microns away from the cells by the end of the culture period. These experiments strongly support the usefulness of OPF hydrogels as MSC carriers for bone regeneration and indicate that hydrogel material properties can influence differentiation of encapsulated progenitor cells.
**Table 6.1:** Change in pH (from control) of initiator components. Data are presented as means ± standard deviation, n = 3.

<table>
<thead>
<tr>
<th>Compound*</th>
<th>ΔpH</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>-1.05 ± 0.01</td>
</tr>
<tr>
<td>TEMED</td>
<td>1.69 ± 0.02</td>
</tr>
<tr>
<td>APS/TEMED</td>
<td>0.74 ± 0.03</td>
</tr>
</tbody>
</table>

*Concentration of each component was 25 mM; experiments performed in MSC culture media.*
**Table 6.2:** Number- ($M_n$) and weight-average ($M_w$) molecular weight of synthesized oligomers as determined by gel permeation chromatography. Data are presented as means ± standard deviation, $n = 3$.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>MW of PEG</th>
<th>MW of Oligomer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$M_n$</td>
<td>$M_w$</td>
</tr>
<tr>
<td>OPF 10K</td>
<td>13,650 ± 860</td>
<td>15,710 ± 970</td>
</tr>
<tr>
<td>OPF 3K</td>
<td>4040 ± 210</td>
<td>4330 ± 220</td>
</tr>
</tbody>
</table>
**Table 6.3:** Times to onset and completion of gelation (as determined by rheometry*) for OPF 10K and 3K with the radical initiators APS/TEMED (25 mM). Data are presented as means ± standard deviation, n = 3.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Onset (min)</th>
<th>Completion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPF 10K</td>
<td>3.6 ± 0.5</td>
<td>15.4 ± 0.8</td>
</tr>
<tr>
<td>OPF 3K</td>
<td>3.6 ± 0.2</td>
<td>34.0 ± 4.1</td>
</tr>
</tbody>
</table>

*Experiments performed in phosphate-buffered saline.
**Table 6.4:** Fold swelling and theoretical sol fraction for OPF 10K and 3K hydrogels crosslinked with APS/TEMED*. Data are presented as means ± standard deviation, n = 3.

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Fold Swelling</th>
<th>Sol Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPF 10K</td>
<td>17.5 ± 0.2</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>OPF 3K</td>
<td>13.4 ± 0.4</td>
<td>0.38 ± 0.02</td>
</tr>
</tbody>
</table>

*Experiments performed in distilled, deionized water.*
**Figure 6.1:** Fold swelling (A) and sol fraction (B) of OPF hydrogels without cells over 28 days at 37°C in cell culture media (± standard deviation, n = 3-4). There was significantly greater swelling for the OPF 3K hydrogels at days 21 and 28 than at days 1 and 7.
Figure 6.2: Cell number per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3-4). * indicates that, at day 28, 10K- samples had significantly fewer cells than the same sample type at the previous time points, and fewer cells than the other treatments for this time point (p ≤ 0.05).
**Figure 6.3**: Alkaline phosphatase activity per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3-4). * indicates that, at day 28, 10K+ and 3K+ samples demonstrated significantly higher ALP levels than for the same sample types at previous time points (p ≤ 0.05).
**FIGURE 6.4:** Cumulative osteopontin secretion per sample for OPF-MSC constructs over 28 days in culture (± standard deviation, n = 3). A) All sample types. B) Samples cultured without dexamethasone (10K- and 3K- only).
**Figure 6.5:** Calcium content per sample for OPF-MSC constructs after various days in culture (± standard deviation, n = 3-4). * indicates that these constructs showed significantly higher calcium deposition compared with the same sample type on day 1 or day 7. # indicates that the 10K+ samples exhibited more calcium accumulation than 10K+ constructs at days 1 and 7 and than 3K+ samples at days 21 or 28 (p ≤ 0.05).
**Figure 6.6:** Calcium content per sample for 10K OPF-MSC constructs after 21 and 28 days in culture, depicted with reference to particular cell isolation. A) Results from the two isolations cultured without dexamethasone. B) Results from the two isolations cultured with dexamethasone.
**Figure 6.7:** OPF-MSC constructs after 7 and 21 days of *in-vitro* culture. Polymer is labeled P, mineralized matrix is labeled M and arrows indicate the location of cells. All 10K and 3K samples looked similar to A) and B), respectively, at day 1. Hydrogels with cells from both isolations cultured without dexamethasone (I-1 and I-2) appeared similar to C) by day 21. D) and G) represent 10K+ hydrogels with cells from I+1 at day 21. E) and H) represent 10K+ hydrogels with cells from I+2 at day 21. F) and I) represent cells from either isolation (I+1 or I+2) used in 3K+ samples at day 21. Magnification for A-F is 200X, for G-I 400X. Scale bar represents 100 μm.
**Figure 6.8:** OPF-MSC constructs after 28 days of *in-vitro* culture. A) and B) represent 10K- hydrogels that demonstrated low and high amounts of calcium deposition, respectively. C) and D) represent 3K- hydrogels that demonstrated low and high amounts of calcium deposition, respectively. E) depicts 10K+ samples (cells for these samples came from I+2). F) depicts 3K+ samples (cells for these samples came from I+2). Magnification is 200X. Scale bar represents 100 μm.
CHAPTER VII

CONCLUSIONS

The overall goal of this work was to develop and characterize synthetic hydrogel materials based on oligo(poly(ethylene glycol) fumarate) (OPF) as injectable cell carriers for orthopaedic tissue engineering. Several specific objectives, including formation and characterization of OPF hydrogels with varied swelling and mechanical properties, characterization of the cytotoxicity of these hydrogels leading to modification of the crosslinking formulation for maximal cytocompatibility, and characterization of the effect of changes in hydrogel swelling properties and media supplements on osteogenic differentiation of rat bone marrow stromal cells (MSCs) encapsulated in OPF hydrogels in vitro, were identified and investigated as a part of this project.

Towards the first objectives, studies demonstrated that alteration of the poly(ethylene glycol) molecular weight used in the synthesis of the OPF macromer led to controlled changes in swelling and mechanical properties of the resulting crosslinked gels. These hydrogels were characterized in both the swollen and dry states, leading to calculation of their mesh size, which ranged from 76 ± 2 Å to 160 ± 6 Å. In addition, it was found that these gels could be laminated during crosslinking. Thus, layered constructs could be formed, with each layer having distinct mechanical properties. This may provide a unique method to tissue engineer composite replacements for complex orthopaedic tissues, such as those found in osteochondral defects in the knee joint.

As the next stage in the development of injectable cell-hydrogel constructs, the
cytotoxicity each component of the OPF formulation, including the radical initiators and their derivatives, was characterized. From these studies, it was found that the overall pH of the formulation, as well as length of exposure to the components, had significant effects on viability of rat MSCs. Using this information, an alternative initiator system was identified which remained near neutral pH in cell culture media and resulted in crosslinking of two types of OPF hydrogels in 8 min at 37°C. This is the first known report describing curing of synthetic hydrogels with embedded cells based on temperature (rather than exposure to light) and, therefore, could be extremely useful for a variety of clinical applications, including those deep within the body where light penetration is limited.

The optimized OPF formulations were then used to investigate effects of changes in hydrogel swelling properties and media supplements on osteogenic differentiation of encapsulated rat MSCs. After 28 days of in vitro culture, evidence of cellular differentiation was found in all sample types, indicating that the encapsulation procedure did not have a detrimental effect on the ability of these MSCs to form bone-like tissue. Calcified matrix was observed in the hydrogels both with and without the presence of osteogenic media supplements (dexamethasone). In the presence of supplements, OPF hydrogels with greater swelling promoted embedded MSC differentiation over those that swelled less. In all samples by day 28, areas of mineralized matrix were obvious many microns away from the cells, indicating that the hydrogel mesh size was large enough to allow diffusion of matrix components throughout the material.

The work compiled in this thesis has demonstrated the great potential of OPF hydrogels as injectable cell carriers for orthopaedic tissue engineering. Based on
alterations to the chemical structure of the OPF molecule during synthesis, controlled crosslinked networks can be formed. These networks result in a variety of possible hydrogel material properties, which can, in turn, affect differentiation of embedded progenitor cells. The flexibility of this OPF-based system, its ability to be thermally crosslinked at low temperatures, and the possibility of forming well-defined laminated constructs provide significant advantages for its use in cell delivery for a variety of tissues. The development of such a system is an important first step toward the ultimate goal of restoring complex orthopaedic defects in a minimally-invasive manner.
CHAPTER VIII

BIBLIOGRAPHY


129. Martens PJ, Bryant SJ, Anseth KS. Tailoring the degradation of hydrogels formed from multivinyl poly(ethylene glycol) and poly(vinyl alcohol) macromers for cartilage tissue engineering. Biomacromolecules 2003;4:283-292.


153. Jabbari E, Behravesh E, Mikos AG. Development of a biodegradable redox initiated oligo(PEG fumarate)-based hydrogel as an *in situ* crosslinkable cell carrier. AIChE Conference; 2002 November, Indianapolis, IN; Abstract 189H.


